



1996

Regulation of Sarcoplasmic Reticulum Calcium Atpase (Serca2) Gene Expression in Left Ventricular Hypertrophy and Heart Failure

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LOYOLA UNIVERSITY CHICAGO

REGULATION OF SARCOPLASMIC RETICULUM CALCIUM ATPase (SERCA2)
GENE EXPRESSION IN LEFT VENTRICULAR HYPERTROPHY AND HEART
FAILURE

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGY

BY

MING QI

CHICAGO, ILLINOIS

JANUARY 1996

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ACKNOWLEDGMENTS

My deepest appreciation goes to Allen M. Samarel, M.D. for his guidance, patience and encouragement throughout this thesis work. I would also like to express sincere gratitude to my committee members, Drs. Donald M. Bers, David E. Euler, Stephen L. Lipsius and Kaie M. Ojamaa for their selfless time and effort, expert advice and valuable criticisms. The technical guidance offered by Dr. Ojamaa, Dr. Euler and Dr. Leanne Cribbs is greatly appreciated.

My gratitude is extended to Dr. Pamela Lucchesi for using her precious time to read and critique my thesis. Lisa Spragia, Alan Ferguson, Li Zhang, Peggy Richied, Dr. Qing Fan and Dr. Elias Eleftheriades, trusted and treasured friends, offered technical support and help in many other ways along my journey toward the Ph.D. degree.

I also would like to thank all the faculty members, staff and graduate students in the Department of Physiology for their assistance and support throughout the course of my training.

Finally, enormous thanks go to my: Dad, Mom, mother and father in-law who have always been there for me. Words cannot express how much has meant to me. Lastly, but most importantly, I thank Zhaogang, my loving husband for his infinite patience. He understood the reasons for the long hours required to complete my research, and tolerated my time away without complaint.

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LIST OF ABBREVIATIONS

Act-D	actinomycin-D
aFGF	acidic fibroblast growth factor
ALC	atrial type myosin light chain
ANF	atrial natriuretic factor
AngII	angiotensin II
ANOVA	analysis of variance
AoC	aortic corarctation
ATP	adenosine triphosphate
β -gal	β -galactosidase
BDM	2,3-butanedione monoxime
bFGF	basic fibroblast growth factor
cAMP	cyclic adenosine monophosphate
$[Ca^{2+}]_i$	intracellular calcium
CaMK	calmodulin-dependent protein kinase II
CaPF1	Ca^{2+} ATPase promoter factor-1
CI	contractility index

CHF	congestive heart failure
DAP	diastolic blood pressure
DAG	diacylglycerol
DMEM/F12	Dulbecco' modified Egel's medium nutrient mixture/F-12 Ham
DTT	dithiothreitol
EB	ethidium bromide
E-C	excitation-contraction
ECM	extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
E-P	phosphrylated intermediate
FFA	unsaturated fatty acid
GIT	guanidine thiocyanate
HBSS	hands' balanced salt solution
HF	Heart failure
Hpro	hydroxyproline
IE	immediate-early
LV	left ventricle
LVEDP	LV end-diastolic pressure
LVH	left ventricular hypertrophy
LysoPC	lysophosphatidylcholine
MAP	mean arterial pressure
MAP	mitogen-activated protein

MARCKS	myristoylated alanine-rich PKC substrate
MF	myofibers
MHC	myosin heavy chain
MITO	mitochondria
MLC	myosin light chain
MOPS	3-[N-morpholino]propane sulfonic acid
ONPG	o-nitrophenyl- β -galactopyranoside
PA	pulmonary artery
PCR	polymerase chain reaction
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PS	phosphatidylserine
RSV	Rous sarcoma virus
RT	relaxation time constant
RT-PCR	reverse transcription polymerase chain reaction
SAP	systolic arterial blood pressure
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SRE	serum response element
SRF	serum response factor
SERCA2	sarcoplasmic reticulum calcium ATPase
SHR	spontaneous hypertensive rat

SL	sarcolemma
SR	sarcoplasmic reticulum
SR Ca ATPase	sarcoplasmic reticulum calcium ATPase
τ	time constant for isovolumic relaxation
TGF- β	transforming growth factor type β
TRE	thyroid hormone responsive elements
T ₃	3, 5, 3', - triiodothyronine
UPE	upstream promoter element
VLC	ventricular type myosin light chain
V _{max}	maximum velocity

CHAPTER I

INTRODUCTION

Left ventricular hypertrophy (LVH) in response to hemodynamic overload is associated with increased morbidity and mortality due to progressive left ventricular systolic dysfunction, impaired diastolic relaxation, irreversible muscle injury and the ultimate development of congestive heart failure (CHF). Whereas a great deal has been learned over the past 10 years regarding the mechanisms leading to the development of LVH (Chien et al., 1991), little is currently known about the cellular and molecular events responsible for the transition from apparently stable hypertrophy to global left ventricular dysfunction (LeJemtel et al., 1993).

One third of all patients with CHF have LV diastolic dysfunction without significant systolic dysfunction (Fouad, et al., 1984). The mechanisms responsible for the diastolic dysfunction in LVH and CHF are largely unknown, but are likely to be multiple. These may include changes in dynamic factors which modulate the inactivation of systolic force (such as alterations in intracellular calcium ($[Ca^{2+}]_i$) handling during myocardial relaxation), and changes in the composition of the LV myocardium which affect LV compliance (such as tissue fibrosis).

Myocardial relaxation is in part dependent upon the activity of the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2), which is a membrane enzyme responsible for pumping $[\text{Ca}^{2+}]_i$ back into the sarcoplasmic reticulum (SR) during diastole. In both humans and experimental animals with pressure overload LVH and CHF, SERCA2 mRNA and protein levels have been found to be significantly decreased (Komuro et al., 1989, de la Bastie et al., 1990, Levtsky et al., 1991, Arai et al., 1993, Hasefuss et al., 1994 and Matsui et al., 1995). A decrease in SERCA2 activity would be expected to produce prolongation of the $[\text{Ca}^{2+}]_i$ transient, and secondarily to cause delayed myocardial relaxation. Therefore, it is conceivable that the observed reduction in SERCA2 activity may play a causal role in the development of diastolic dysfunction seen in pressure overload cardiac hypertrophy, and may also be involved in the deleterious transition to CHF. It should be pointed out, however, that not all forms of human CHF demonstrate reduced SERCA2 expression. For instance, Movsesian et al (1994) found that SR Ca^{2+} ATPase protein content was not diminished in patients with idiopathic dilated cardiomyopathy and end-stage CHF, indicating that down-regulation of SR Ca^{2+} ATPase is not part of the molecular pathophysiology of this disorder. Thus, the role of SR function in LVH and CHF warrants further investigation. Furthermore, there is no evidence to demonstrate that a decrease in SR Ca^{2+} ATPase activity directly correlates with the deterioration of hemodynamic function that occurs during the development of pressure overload hypertrophy. This is one of the specific aims of this dissertation study.

A great deal of information is currently available about the SR Ca^{2+} ATPase gene family and the SERCA2 gene. It is known that SERCA2 gene expression is tissue-specific and

developmentally regulated (Arai et al., 1992, Lompré 1991, Fisher et al., 1992, Antoon et al., 1995, Mnger et al., 1994, Mahony et al., 1986). However, little is known about the molecular mechanisms involved in the regulation of SERCA2 gene expression. Thyroid hormone is the only well studied factor known to regulate cardiac SERCA2 gene expression at the transcriptional level (Arai et al., 1991, Nagai et al., 1989, Rohrer et al., 1988, 1991, Ojamaa et al., 1992, Zarain-Herzberg et al., 1994). Interestingly, the reduced SERCA2 mRNA levels observed in pressure-overloaded, hypertrophied hearts occurs in the presence of normal circulating levels of thyroid hormones, indicating that other factors (including a direct effect of mechanical load) may regulate SERCA2 gene expression *in vivo*. At the present time, it remains largely unknown which signal transduction pathways are involved in the regulation of SERCA2 gene expression during LVH progression.

Obviously, it is difficult to precisely identify the manner in which mechanical stimuli regulate SERCA2 gene expression in the pressure overloaded and failing heart *in vivo*. It is even more complicated to identify second messengers that may be involved in transducing mechanical signals from the cell surface to the nucleus during the development of pressure overload LVH. To understand the mechanisms by which hemodynamic overload alters the expression of cardiac genes, pressure overload hypertrophy *in vivo* has been mimicked by stimulation of cultured cells *in vitro* with hormonal (Simpson et al., 1991), humoral (Qi et al., 1994, Parker et al., 1990), or mechanical factors (Samarel and Engelmann, 1991, Komuro et al., 1991). Primary cultures of neonatal ventricular myocytes have been widely used to identify the signal transduction pathways involved in the development of cardiac hypertrophy. A growing body of evidence has demonstrated that protein kinase C (PKC)

(Kariya et al., 1991, Shubeitha et al., 1992, Samarel and Engelmann, 1991, Qi et al., 1994) plays a very important roles in the regulation of cardiac gene expression during myocyte hypertrophy. Other signal transduction pathways such as Ca^{2+} /Calmodulin-dependent protein kinase II (CaMK) may also be involved in regulating gene expression in cardiac hypertrophy (McDonough and Glembotski, 1992, 1994). Whether these factors are also involved in the regulation of SERCA2 gene expression during pressure overload hypertrophy is not known, but is specifically addressed in this dissertation work.

The main focus of my dissertation work is to evaluate the role of SERCA2 gene expression in the development of pressure overload LVH and CHF and to determine the role of mechanical activity and PKC in the regulation of SERCA2 gene expression. These aims are accomplished by experiments at three levels of investigation.

Firstly, I developed an animal model of pressure overload LVH (abdominal aortic coarctation (abdominal AoC) in Sprague-Dawley rats) that could be used to evaluate SERCA2 gene regulation during the transition from compensated LVH to decompensated CHF. This transition period was identified by hemodynamic indices of LV systolic and diastolic function measured in the intact animal. Hemodynamic measurements were then correlated with alterations in LV tissue composition (including the level of expression of SERCA2 mRNA and protein) in the hypertrophied myocardium.

Secondly, primary cultures of neonatal rat ventricular myocytes were used to study the potential cellular and molecular mechanisms responsible for the regulation of SERCA2 gene expression during cardiac myocyte hypertrophy and atrophy. I examined the effects

that alterations in mechanical activity and PKC activity have on myocyte growth and the expression of SERCA2 mRNA and protein levels in these cultured heart cells.

Finally, to further investigate the molecular mechanisms involved in SERCA2 gene expression, a transient transfection assay and message stability analysis were used to determine whether the changes in SERCA2 mRNA levels induced by changes in mechanical activity and PKC activity were mediated by changes in the rate of SERCA2 gene transcription or were due to changes in SERCA2 mRNA stability.

Thus, the combination of a variety of approaches provides us with additional useful information about the regulation of SERCA2 gene expression during LVH and the transition to CHF.

CHAPTER II

REVIEW OF RELATED LITERATURE

A. INTRODUCTION

According to the Framingham Study, a 30-year longitudinal study of cardiovascular risk among residents of Framingham, Massachusetts, arterial hypertension is the single most important etiologic factor in the development of symptomatic CHF. Furthermore, the risk of developing CHF increases 6-18 fold in the presence of electrocardiographic and/or echocardiographic evidence of LVH (Kannel et al., 1987). Thus the development of LVH in response to arterial hypertension and other forms of hemodynamic overload is a harbinger for the subsequent development of significant cardiovascular morbidity and mortality. However, the changes which trigger the transition from stable LVH to overt CHF are poorly characterized.

In response to increased hemodynamic load, the heart alters its function, increasing its muscle mass and qualitatively and quantitatively changing its gene expression. It is apparent that one of the alterations in cardiac gene expression that accompanies the hypertrophic process is a reduction in the expression of the SR Ca ATPase (SERCA2)

mRNA and protein levels of this calcium transporter are down-regulated during pressure overload hypertrophy and CHF in a variety of experimental animal models of hemodynamic overload, and in human CHF. This decrease in SERCA2 gene expression may be at least in part the cause of the abnormal Ca^{2+} handling and slowed cardiac relaxation in pressure overloaded and failing hearts. To date, a thorough investigation of SERCA2 gene regulation and the potential cellular and molecular mechanisms involved in its regulation during LVH progression have not been conducted.

This chapter is designed to furnish the reader with a basic understanding of the physiology and molecular biology of cardiac hypertrophy, and with information regarding the molecular biology of SR Ca ATPase and its regulation during pressure overload hypertrophy and CHF. Literature regarding the use of cell culture models of cardiac hypertrophy is also reviewed, as these cell culture systems have greatly aided our understanding of potential triggers for the development of cardiac hypertrophy. In general, two types of triggers involved in the development of pressure overload hypertrophy *in vivo* have been intensively investigated in cultured cells. These are mechanical triggers such as passive stretch and active contraction; and trophic triggers such as peptide-derived growth factors (including fibroblast growth factors, transforming growth factors, angiotensin II, and endothelin), and adrenoceptor activation. The accumulated data suggest the involvement of PKC-dependent signaling pathways in the transduction of both mechanical and humoral signals leading to myocyte hypertrophy. A growing body of evidence has demonstrated that several isoenzymes of PKC can be activated by different hypertrophic stimuli. Therefore in

this chapter, the structure and function of the various cardiac PKC isoenzymes are also reviewed in the context of their potential role in cardiac hypertrophy signal transduction.

B. HEMODYNAMIC OVERLOAD INDUCED CARDIAC HYPERTROPHY

1. General Concept of Cardiac Hypertrophy.

During postnatal development, the cardiac myocyte rapidly loses its ability to proliferate, and subsequent growth of the heart is dependent upon enlargement of pre-existing muscle cells. Under normal conditions, an equilibrium is attained in which cardiac muscle mass matches the workload that is imposed on the heart. When subjected to a chronic increase in hemodynamic load (in the form of either pressure or volume overload), the myocardium adapts by increasing its muscle mass. For reasons incompletely understood, there appears to be a limit to this compensatory response, and long-standing hypertrophy can often result in the subsequent development of CHF, which is a pathophysiological condition characterized by the inability of the heart to pump blood at a rate commensurate with the requirements of the metabolizing tissues. Therefore, LVH is a common consequence of hypertension and other forms of hemodynamic overload, and LVH is an independent risk factor for the subsequent development of cardiovascular morbidity and mortality.

LVH is characterized by myocyte hypertrophy and hypertrophy/hyperplasia of nonmyocyte cells. Myocyte hypertrophy is cell enlargement accompanied by a generalized increase in protein content per cell. This generalized increase in cellular protein content results from an overall increase in the rate of protein synthesis, which is associated with increases in the amounts of both messenger and ribosomal RNA. The increase in total RNA content is based on increases in the rate of DNA transcription as well as RNA stability (McDermott et al., 1989). Cardiac hypertrophy is also characterized by activation of a new program of gene expression. These genes can be divided into two classes: immediate-early (IE) response genes and late-response genes. During the last decade, a wealth of information has been obtained concerning the transient expression of IE genes in the heart in response to work overload. IE genes include a number of proto-oncogenes, which encode an intricately inter-connected network of proteins whose function is also crucial to normal growth and differentiation (Bishop, 1987, Nulvagh et al., 1988, Weinberg, 1989, Schneider et al., 1991). An example of IE gene induction is the enhanced expression of *c-fos* and *c-myc* in the rat heart *in vivo* in response to thoracic aortic banding (Komuro et al., 1988). These IE genes encode nuclear transcription factors, whose own transcription requires no new protein synthesis. The induction of proto-oncogene transcription may initiate the earliest steps in altered gene expression during hemodynamic overload. In addition, there is increased expression of a number of stress proteins in the heart in response to hemodynamic overload (Delcayre et al., 1988), whose function in the hypertrophic response remains obscure.

In contrast to the relatively transient expression of proto-oncogenes, cardiac myocytes respond to hemodynamic overload with a sustained alteration in the expression of many late-response genes, including the cardiac contractile protein genes. The contractile unit of the myocardium is the sarcomere, which is composed of seven major proteins and several minor ones. All of these major proteins have multiple isoforms, and the transcription rates of some isoforms are selectively increased in cardiac hypertrophy. In general, this response is characterized by the re-expression of contractile protein isoforms which are predominantly expressed in the immature heart (Nadal-Ginard and Mahdavi, 1989, Morkin, 1987, and Schiaffino et al., 1989.). As a late-response gene, cardiac myosin heavy chain (MHC), is best studied both at the molecular and tissue levels (for review see Nadal-Ginard and Mahdavi, 1989). In adult rodents and other small mammals, cardiac myocytes predominantly express a cardiac-specific, fast MHC isoform (MHC- α). In response to hemodynamic overload, the cardiac/slow skeletal muscle isoform (MHC- β) is induced (Mercadier, et al., 1981, Klein et al., 1992). Of note, MHC- β is normally expressed at high levels only in the fetal and neonatal heart. Actin, another important cardiac contractile protein, also exists as 3 isoforms (skeletal, cardiac and smooth muscle α -actin). In pressure overloaded rat heart, the skeletal α -actin isoform is up-regulated, with unaltered or decreased cardiac α -actin expression (Schwartz et al., 1986, 1993 and Izumo et al., 1988). Since skeletal α -actin is highly expressed in utero, its re-expression during pressure overload represents another example in which a “fetal program” of gene expression is reactivated by hemodynamic overload. Another actin isoform, smooth α -actin normally

expressed in vascular smooth muscle, was found to be reactivated with pressure overload hypertrophy, and the high levels of smooth muscle actin mRNA in the hypertrophied myocardium strongly suggest that this isoform is present in the myocytes (Black et al., 1991). The two other types of cardiac myosin subunits, myosin light chains (MLC) 1 and 2, both exist as two isoforms, atrial and ventricular. The atrial isoform ALC1 increases in several forms of ventricular hypertrophy (Boheler and Schwartz, 1992), whereas the ventricular types VLC1 and VLC2 are re-expressed in pressure-overloaded human atria (Cummins, 1982). This reprogramming of cardiac myocyte gene expression by hemodynamic overload is not limited to contractile proteins. Ventricular myocytes also re-express atrial natriuretic factor (ANF), a gene whose expression is lost during normal postnatal cardiac development (Chien et al., 1991, Lattion et al., 1986, Knowlton et al., 1991). In contrast to these up-regulated genes, mRNA levels encoding some genes, including the gene encoding the SR Ca ATPase, are down-regulated in response to pressure overload. Evidence documenting that SERCA2 mRNA and protein levels decrease during pressure overload hypertrophy and heart failure are reviewed in a separate section of this chapter.

The muscle cell compartment of myocardial tissue is not the only cell type that is affected during pressure overload. Hypertrophy and hyperplasia of nonmyocyte components also occurs in pathological forms of cardiac hypertrophy. The increased expression of collagen genes and remodeling of the cardiac extracellular matrix is another characteristic of

overload-induced cardiac hypertrophy, and these alterations may also contribute to the deterioration of cardiac function in the hypertrophied and failing heart.

2. *In Vivo* Animal Models.

Many models of cardiac hypertrophy produced in the intact experimental animal have been used to simulate the human condition. Each model has unique advantages as well as disadvantages which are relevant to the interpretation of the acquired experimental data (Table 1). Characteristics of the growth responses vary widely, depending on experimental conditions. Variables commonly recognized are the severity, duration and type of overload (i.e., pressure vs. volume), and the rate at which the hemodynamic overload is applied (i.e., acute vs. gradual), as well as the age and species of the animal. A major complication in the study of *in vivo* models of pathological cardiac hypertrophy is the change in the body weight of the animal. Normal growth can be affected by surgical trauma, nutritional, and other factors. To evaluate the degree of hypertrophy, in such cases, one must rely not on the size of the heart but on its ratio to body size. The second complication arises when the overload is accompanied by injury to the blood vessels and heart. Coarctation of either the thoracic and abdominal aorta in juvenile animals are the most commonly used methods to produce pressure overload LVH. Both surgical approaches produce increased LV afterload, induce substantial LVH, and generate the pressure-overload phenotype which simulates to some extent LVH in humans. Abdominal aortic coarctation (AoC) but not thoracic AoC is associated with systemic hypertension (Weinberg et al., 1994). Whereas abdominal AoC

produces no cardiac myocyte necrosis, thoracic AoC can cause severe muscle damage, and is also associated a higher perioperative mortality. Therefore, abdominal AoC is a better model to mimic chronic human hypertension. However, thoracic AoC has the advantage that hypertension-induced release of neurohumoral agents, growth factors, and other substances into the circulation from noncardiac tissues is limited or prevented.

TABLE 1

In vivo animal models for studying hemodynamic overload induced cardiac hypertrophy.

Pressure overload	Volume overload
<p>Aortic stenosis</p> <p>Thoracic aortic coarctation</p> <p>(rat, Takahashi et al., 1992), (ferret, Wang et al., 1994)</p> <p>(cat, Bailey & Houser, 1992)</p> <p>Abdominal aortic coarctation</p> <p>(rat, Nagai et al., 1989) (pig, Dhalla et al., 1984)</p> <p>(guinea pig, Cory et al., 1994)</p>	<p>Arteriovenous fistula</p> <p>(rat, Dart and Holloszy, 1969)</p>
<p>Pulmonary artery stenosis</p> <p>(cat, Schlafer et al., 1978), (rat, Julian et al., 1981), (rabbit, Matsui et al., 1995), (ferret, Gwathmey & Morgan, 1985)</p>	<p>Bradycardia</p> <p>(dog, Brockman, 1965)</p>
<p>Hypertension</p> <p>Renal ischemia (rat, Goldblatt et al., 1934) (guinea pig, Naqvi and Macleod, 1994)</p> <p>Nephrectomy (rat, Grollman, 1944)</p> <p>Nephrectomy+DOCA+salt (rat, Bartsova et al., 1969)</p> <p>Aldosterone+salt (rat, Garwitz and Jones, 1981)</p> <p>Spontaneous SHR (rat, LeJemtel et al., 1993b)</p> <p>Spontaneous, Dahl, salt-sensitive (rat, Pfeffer et al., 1984)</p>	<p>Sideropenic anemia</p> <p>(rat, Bartsova et al., 1969)</p> <p>Aortic insufficiency</p> <p>(rabbit, Hatt et al., 1970)</p>

C. MECHANISMS RESPONSIBLE FOR THE CHANGES IN CARDIAC PERFORMANCE DURING THE DEVELOPMENT OF PRESSURE OVERLOAD LVH

Functional abnormalities in myocardial contraction and relaxation are commonly observed in patients with LVH due to hypertensive and valvular heart diseases, and these abnormalities have been successfully reproduced in a variety of experimental animal models of pressure overload LVH. These include a reduction in the velocity of contraction, prolonged ejection, and delayed relaxation (Capasso et al., 1990). Impaired contractile function can also be demonstrated in isolated cardiac muscle strips and perfused heart preparations from animals with experimentally induced pressure overload LV or RV hypertrophy (Bing et al., 1971, Alpert and Mulieri, 1982, Bentivegna et al., 1991). These abnormalities include prolongation of isometric contraction, an increase in the time to peak tension development, a decrease in isotonic shortening velocity, and decreased maximum isometric force. There is now considerable evidence to indicate that ventricular diastolic properties are also impaired in experimental animals and humans with pressure overload (Fouad, 1987, Lorell et al., 1990, Siri et al., 1989, Swynghedauw et al., 1992). Abnormalities in left ventricular diastolic function can be broadly classified in two groups: (1) those related to left ventricular relaxation and early diastolic filling; and (2) those related to altered passive pressure-volume characteristics and reduced compliance. Since the extent of cardiac muscle activation with each systole is dependent on the completeness of

relaxation during the preceding diastole; and ventricular systolic function is dependent upon adequate ventricular diastolic filling, decreased diastolic function may thus contribute to the systolic abnormalities in the pressure-overload hypertrophied heart.

1. MHC Isoenzyme Switching is Partly Responsible for Contractile Dysfunction During Pressure Overload Hypertrophy.

The early search for biochemical correlates of cardiac hypertrophy centered around attempts to relate myosin ATPase activity to physiological function. Early studies using rodent models of LVH demonstrated a close correlation between the maximum velocity of contraction (V_{\max}) and the specific activity of the Ca^{2+} activated, myosin ATPase (Pope, et al., 1980, Schwartz, et al., 1981). Subsequent studies revealed that cardiac myosin heavy chain (MHC) was composed of two different isoforms, which are the products of two closely linked genes. MHC- α , which has high Ca^{2+} and actin-activated ATPase activity, is associated with a fast shortening velocity. In contrast, MHC- β , which has lower ATPase activity, is associated with slower shortening velocity. MHC- α is the predominant MHC isoform expressed in the ventricular tissue of normal adult rodents and other small mammals, whereas MHC- β is highly expressed in the fetal and neonatal heart (Brandl, et al., 1986, Lompre et al., 1984). In humans and other large mammals, MHC- α is only predominant shortly after birth, with MHC- β the isoform predominantly expressed during both fetal and adult life (Lompre et al., 1981 and Chizzonite, et al., 1984.). The situation differs in the atria where MHC- α is the predominant isoform throughout life in both small

and large mammals (Swynghedauw, 1986, Lompre et al., 1984 and Chizzonite, et al., 1984). In the adult rat, acute LV pressure overload leads to the re-induction of the fetal isoform (MHC- β) and suppression of the high ATPase MHC- α isoform (Chassagne and Schwartz, 1993, Imamura et al., 1991, Izumo et al., 1987, Schwartz et al., 1992). The "isoform switch" from MHC- α to MHC- β results in a slower rate of ATP cycling by myosin, which in part accounts for the decrease in the velocity of fiber shortening observed in the pressure-overloaded rat heart. However, this isoform switch cannot explain reduced contractile function in humans and other large mammals with pressure-overload LVH, since MHC isoenzyme switching does not occur (Hoffmann and Grisk, 1986, Mercadier et al., 1983). Thus other mechanisms must also be responsible for the slowed contraction observed in LVH and CHF. In large mammalian ventricles, $[Ca^{2+}]_i$ transients are likely to be the only determinant of V_{max} . Therefore, the membrane protein components responsible for changes in $[Ca^{2+}]_i$ may be the main determinants of changes in V_{max} that occur in pressure overload LVH.

2. Myocyte $[Ca^{2+}]_i$ Mishandling During LVH and CHF.

It is well established that contraction in cardiac muscle is initiated by an increase in $[Ca^{2+}]_i$. Ca^{2+} enters the cell during the action potential via sarcolemmal Ca^{2+} channels (and possibly also via Na/Ca exchange). Some of this Ca^{2+} contributes to direct activation of the myofilaments, but Ca^{2+} influx also triggers the release of Ca^{2+} from an SR storage pool via a process referred to as Ca-induced Ca release. The released and activator Ca^{2+} then binds to

a component of the contractile apparatus (troponin C) which initiates actin-myosin cross-bridge formation and muscle contraction. For relaxation to occur, Ca^{2+} must be removed from the cytoplasm, allowing Ca^{2+} to dissociate from the myofilaments. There are several systems in the cardiac muscle cell which contribute to $[\text{Ca}^{2+}]_i$ decline during cardiac relaxation: (1) Ca^{2+} can be transported back into the SR by the SR Ca ATPase; (2) Ca^{2+} can be transported across the sarcolemma, either in exchange for Na^+ (i.e. via the activity of the Na-Ca exchanger), or by the activity of the sarcolemmal Ca-ATPase; or (3) Ca^{2+} can be sequestered within mitochondria (Figure 1) (See Bers, 1991 for detailed review). The SR Ca ATPase and Na-Ca exchanger are by far the most dominant mechanisms, and the relative contributions of these systems varies in different cardiac muscle preparations (Hove-Madsen and Bers, 1993, Bassani et al., 1994 a). In adult rat LV myocytes, the relative contribution of the various processes to $[\text{Ca}^{2+}]_i$ decline were estimated as: SR 87%, mitochondria 1.7%, Na-Ca exchanger 8.7%, sarcolemmal Ca-ATPase 2.6% (Negretti et al., 1993). Because $[\text{Ca}^{2+}]_i$ plays a central role in the process of cardiac excitation-contraction coupling, $[\text{Ca}^{2+}]_i$ mishandling may provide the basis for both systolic and diastolic dysfunction in pressure overload. Morgan's group has shown that the prolonged isometric contraction of cardiac tissue from animals with cardiac hypertrophy and humans with CHF correlates with a similar prolongation of the calcium transient (Bentivegna et al., 1991, Gwathmey et al., 1987, Gwathmey and Morgan, 1985, Bing et al., 1991). For instance, the prolonged relaxation-phase of the isometric twitch observed in ferret right ventricular pressure overload hypertrophy was associated with a markedly slowed decay of $[\text{Ca}^{2+}]_i$, as

detected with aequorin (Gwathmey and Morgan, 1985). Prolonged $[Ca^{2+}]_i$ decline appears to predominantly reflect the inability of the SR to sequester Ca during diastole. Although slowed $[Ca^{2+}]_i$ sequestration contributes to diastolic relaxation abnormalities, it is probably not the only factor responsible for slowed relaxation. A component of the impaired diastolic relaxation seen in pressure overload hypertrophy is both energy and Ca^{2+} independent, and most likely reflects altered cross-bridge cycling rates and/or changes at the level of the myofilaments (Gwathmey and Morgan, 1993). Furthermore, studies using isolated myocytes from pressure overloaded, hypertrophied hearts have suggested that changes in diastolic relaxation may be due to slowed resequestration of Ca^{2+} , whereas the decreased force generation of these preparations can largely be attributed to a decrease in myofilament Ca^{2+} responsiveness (Wang et al., 1994, Bailey and Houser, 1992, Moore et al., 1991).

In summary, $[Ca^{2+}]_i$ during relaxation is primarily controlled by Ca^{2+} uptake by the SR. The rate of sequestration (and perhaps the rate of release of Ca^{2+} by SR) is impaired in the hypertrophied heart. Clearly, a potential cause for slowed relaxation may be related to the activity of the enzyme largely responsible for SR calcium re-uptake during diastole, namely the SR Ca^{2+} ATPase.

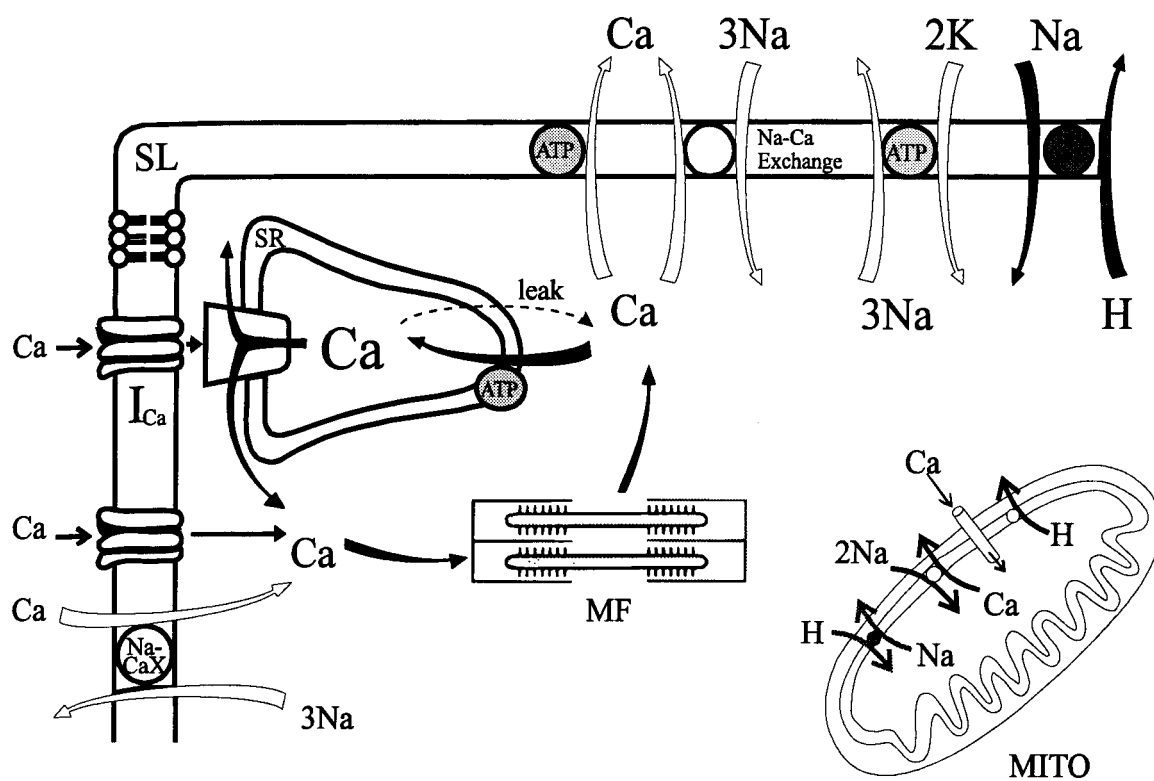


Figure 1: Ca movement in cardiac myocyte (adapted from Bers, 1991). SL = sarcolemma, MF = myofibers, MITO = mitochondria.

3. Changes In The Cardiac Extracellular Matrix (ECM) May Contribute To Myocardial Functional Abnormalities In LVH And CHF.

Another characteristic of myocardial tissue from pressure overloaded hearts is changes in compliance. The major determinant of ventricular tissue stiffness is the structure and concentration of extracellular matrix collagens. The myocardium may be differentiated into compartments occupied by cardiac myocytes and the interstitial space between these cells. The interstitial space is composed of nonmuscle cells (i.e. cardiac fibroblasts and endothelial cells) and a highly organized collagen network comprising the cardiac extracellular matrix (ECM). The ECM serves to coordinate contractile force generated by cardiac myocytes and contributes to the passive stretch characteristics of the ventricles. Furthermore, muscle contraction is believed to compress elements of the cardiac ECM; subsequent expansion of the matrix in diastole has been suggested to provide energy for active re-lengthening of cardiac myocytes (Robinson et al., 1986). Thus deformation of the ECM may help restore ventricular myocytes to precontraction length (Robinson et al., 1986, Fouad et al., 1986). This expansion phase is thought to create suction that pulls blood into the ventricles from the atria during the rapid filling phase of the cardiac cycle. The main ECM proteins are collagen and fibronectin which are synthesized by nonmuscle cells (Ahumada et al., 1981, 1984) and laminin which is synthesized by myocytes (Lungdren et al., 1988). Type I and Type III collagens are the major collagenous proteins secreted by cardiac fibroblasts. They form fibrils which comprise the elaborate weave and strut lattice that surrounds individual cardiac myocytes and capillaries. A substantial increase in

collagen volume fraction has been observed in humans with aortic stenosis (Hess et al., 1981, Schwarz, et al., 1978, Schaper and Schaper, 1983) and hypertension (Pearlman , et al., 1982). Similar changes have been observed in hypertensive, nonhuman primates (Weber et al., 1988) and smaller mammals with experimental or genetic hypertension (Bing et al., 1971, Thiedemann et al., 1983, Pfeffer and Pfeffer, 1985, Bartosova et al., 1969). This accumulation of collagen was noted to be either diffusely distributed throughout the myocardium or confined to the subendocardium, or both, and has been implicated as the cause for the impaired diastolic stiffness (Hess et al., 1981) and pump capacity (Pfeffer and Pfeffer, 1985) of the intact ventricle. Fibrillar collagens contain a unique amino acid (4-hydroxyproline, or Hpro). Quantitative analysis of Hpro in acid hydrolysates of myocardial tissue has been frequently used to assess fibrillar collagen content. A strong positive relationship between cardiac Hpro concentration and resting tension has well-documented (Thiedemann, 1983, Willians, 1982). In addition, Bing et al (1971) noted that the velocity of shortening of hypertrophied left ventricular columnar cavneae muscles (obtained after constriction of the aortic arch) was reduced, and inversely related to the Hpro concentration of the endomyocardium. A study by Jalil et al (1988) showed that the increase in passive stiffness of the hypertrophied myocardium appears to be related to interstitial fibrosis. These findings indicate that increases in collagen content and remodeling of the cardiac interstitium are major determinants of increased myocardial stiffness and therefore, may also play a very important role in diastolic dysfunction during pressure overload LVH and CHF.

D. SR Ca ATPase AND SERCA2 GENE IN HEART

In cardiac muscle, the SR plays a central role in the contraction-relaxation cycle by virtue of its ability to regulate $[Ca^{2+}]_i$. Ca^{2+} release from the SR increases $[Ca^{2+}]_i$ (to a maximal systolic concentration of $10^{-5}M$), thus inducing contraction, whereas Ca^{2+} uptake by the SR reduces $[Ca^{2+}]_i$ (to a minimum concentration of $10^{-7}M$ during diastole), thus causing muscle relaxation. Cardiac pump function is thus tightly controlled by the regulated release and removal of Ca^{2+} by the SR. In view of this key role in excitation-contraction coupling, alterations in SR function would be expected to significantly affect cardiac performance. One of the most important myocyte proteins responsible for E-C coupling is the SR Ca^{2+} ATPase. The SR Ca^{2+} ATPase is a 110 kDa integral membrane protein that functions to transport Ca^{2+} from the cytoplasm into the SR lumen against its electrochemical gradient, requiring the consumption of metabolic energy in the form of ATP. The Ca pump also serves to "load" the SR and, in part, determines the amount of Ca^{2+} that is available for release from the SR to activate myofilaments during subsequent contractions.

1. SERCA2 Gene and SERCA Gene Family

Interest in the role of SR Ca^{2+} ATPase activity in normal muscle function has led to considerable research into the structure and function of this enzyme and its gene. The

cardiac SR Ca ATPase is an E1-E2 type of trans-membrane protein, and belongs to a multigene family (SERCA) which consists of five distinct isoforms encoded by three genes (SERCA1, SERCA2 and SERCA3). The SERCA1 gene is expressed exclusively in fast skeletal muscle and encodes two alternatively spliced transcripts which differ in their COOH termini. The SERCA1 isoforms are developmentally regulated: adult skeletal muscle expresses the SERCA1a isoform, whereas neonatal skeletal muscle expresses the SERCA1b type (Brandl et al., 1986). The SERCA2 gene product is alternatively spliced at the 3' end producing SERCA2a which is present in the SR of cardiac and slow-twitch skeletal muscle (Nagai et al. 1989, Lompré et al., 1989, Van Den Bosch et al., 1994), and SERCA2b, which is expressed in the endoplasmic reticulum of smooth muscle and non-muscle tissues in rat, rabbit and human (de la Bastie et al., 1988, Lytton et al., 1988, 1989, Burk et al., 1989). The SERCA2b isoform is identical to the SERCA2a isoform except for the replacement of the COOH-terminal four amino acids with an extended sequence of 49 amino acids. The functional differences between SERCA2a and SERCA2b can be ascribed to the presence of the last 12 amino acids in SERCA2b (Verboomen et al., 1994). Since the SR Ca ATPase in fast skeletal muscle (SERCA1) and cardiac muscle (SERCA2a) are encoded by two different genes which share 84 % amino acid sequence identity (MacLennan et al., 1985), the different properties of the two gene products may in part account for the different relaxation properties of the corresponding muscle types (Lytton et al., 1992, Wu et al., 1994). A third gene in the SERCA family (SERCA3) is also present in both muscle and non-muscle tissue (Burk et al., 1989).

2. Regulation of SERCA2 Gene Expression in The Heart.

a) Regulation of cardiac SERCA2 gene expression during ontogenesis and aging.

Myocardial function changes during ontogenic development (Hoerter and Lecarpentier, 1984, Hoerter et al., 1981, Chemla et al., 1986) and aging (Capasso et al., 1983, Froehlich et al., 1978, Tate et al., 1990, Lakatta et al., 1987). Some of the developmental and age-associated changes in the mechanical properties of the heart have been hypothesized to result from differences in SR properties. In support of this hypothesis, several studies that have compared SR properties in aged and adult rats have reported a decreased rate of Ca^{2+} uptake and a decreased Ca^{2+} ATPase activity with age (Froehlich et al., 1978, Tate et al., 1990, Narayanan, 1987). In addition, the sensitivity to Ca^{2+} uptake does not vary with age, whereas Ca^{2+} uptake and Ca^{2+} -ATPase activity are lower in fetal than in adult SR (Nakanishi and Jarmakani, 1984, Nayler and Fassold, 1977, Mahony and Jones, 1986, Pegg and Nichalak, 1987, Komuro et al., 1989). More recent studies have demonstrated that myocardial SERCA2a mRNA and protein levels increase dramatically during late fetal and early neonatal life, and they achieve a stable, high level of expression during adulthood in rat, rabbit and sheep (Arai et al., 1992, Lompré, 1991, Fisher et al., 1992, Antoon et al., 1995, Mnger et al 1994, Mahony et al., 1986). These levels only decrease substantially during late senescence (Besse et al., 1993, Maciel et al., 1990, Taffet and Tate, 1993), and during pathological states such as hypothyroidism and severe pressure overload (for a detailed review see below). The expression of the SERCA2a isoform is regulated in a cell-

specific manner. However, the expression of SERCA2b is neither tissue-specific nor developmentally regulated. A very stable, low level of the SERCA2b gene is expressed in the heart throughout life and during pathological states (Anger et al., 1994, Van Den Bosch et al., 1994, Verboomen et al., 1994). Thus, unlike the situation with many of the contractile protein genes, changes in cardiac SERCA2 gene expression during pathological states is not the result of isoform switching (Arai et al., 1992, de la Bastie et al., 1990, Nagai et al., 1989).

b). Regulation of SERCA2 mRNA and SR Ca ATPase expression during pressure overload hypertrophy and heart failure. Alterations in SR Ca uptake have been extensively investigated in hemodynamic overload. A growing body of evidence suggests that Ca^{2+} uptake and SR Ca^{2+} ATPase activity are depressed in hemodynamically overloaded, hypertrophied hearts (Ito et al., 1974, Komuro et al., 1989, de la Bastie et al 1990, Levitsky et al., 1991). Most studies have shown that the functional changes in SR Ca^{2+} uptake are actually caused by a decrease in the amount of SR Ca^{2+} ATPase protein (Komuro et al., 1989, de la Bastie et al., 1990, Levitsky et al., 1991 and Matsui et al., 1995). Others have questioned this finding. For instance, Cory et al (1994) found that reduced in SR Ca^{2+} ATPase activity (as measured by Ca uptake by isolated SR vesicles) was not caused by a decrease in the number of Ca^{2+} pumps, which was observed to be increased by 64% 3 weeks after coarctation of the abdominal aorta in guinea pigs. More recently, molecular studies have demonstrated that SERCA2 mRNA levels are quantitatively decreased in non-failing pressure overloaded hypertrophy models (Komuro et. al., 1989, Nagai et. al., 1989).

Therefore, it has been proposed that reduced SERCA2 gene expression may play an important role in the alterations in $[Ca^{2+}]_i$ handling and abnormal myocardial relaxation observed in CHF. In support of this hypothesis, several groups have demonstrated that SERCA2 mRNA and protein levels were markedly decreased in LV tissue of experimental animals and humans with CHF (Arai et al., 1993, Hasefuss et al., 1994, Mercadier et al., 1990, Studer et al., 1994). In contrast, Movsesian et al (1994) found that SR Ca^{2+} ATPase protein content was not diminished in ventricular tissue from patients with idiopathic dilated cardiomyopathy undergoing cardiac transplantation. These results indicate that down-regulation of SR Ca^{2+} ATPase may not be a part of the molecular pathophysiology of all forms of end-stage human heart failure. Thus, the role of SR function in human heart failure warrants further investigation.

The following issues must be considered when studying SR function in hypertrophied and failing hearts. First, it should be noted that alterations in steady state mRNA levels encoding SERCA2 do not always correlate with changes in SR Ca^{2+} uptake. There is evidence to show that the reduction in SR Ca^{2+} ATPase protein concentrations is less profound than the decrease in SERCA2 mRNA levels (Komuro et al., 1989, Matsui et al., 1995). The other important issue is that alterations in SR function seem to depend on the duration and severity, as well as the type of the hemodynamic overload produced (Table 2). Increased SR Ca^{2+} uptake activity (Cuneo and Genda, 1988) or unaltered SERCA2 mRNA levels. (Boluyt et al., 1994) have been reported in SHR rats with mild and severe LVH. Enhanced SR Ca^{2+} uptake has also been documented in mild pressure overload hypertrophy

(Limas et al., 1980 and Dhalla et al., 1984). Working with pressure-overloaded rats, de la Bastie et al (1990) reported that SERCA2a mRNA and protein levels were not reduced in mild hypertrophy (LV weight/body weight <2.6 g/kg), but were significantly decreased in severe LVH (LV weight/body weight >2.6 g/kg) one month after abdominal AoC. However, in both groups, SR Ca^{2+} ATPase activity was depressed irrespective of the degree of LVH. In contrast, Feldman et al (1993) demonstrated that the degree of hypertrophy did not predict the degree of SERCA2 mRNA down-regulation. SERCA2 mRNA levels were found to be decreased only in animals with clinical signs of CHF and diminished LV systolic function (Table 2). The degree of SERCA2 mRNA down-regulation may also be related to the age of the animals in which pressure overload is initially induced. Takahashi et. al (1992) showed that 5 days after ascending AoC, SERCA2 mRNA levels were severely reduced (by 69%) in 18 month-old (aged) rats, but not in 9-month old adult animals. In addition to the above mentioned complications involved in the studies of the regulation of SERCA2 gene expression during LVH and CHF, there is still no evidence to show a direct link between down-regulation of SERCA2 gene expression and impaired diastolic function in LVH and heart failure. Therefore, in order to prove that decreased SR Ca^{2+} ATPase activity accounts for the defect in Ca handling and slowed diastolic relaxation observed in both human and experimental heart failure, a thorough investigation of functional changes in SR Ca^{2+} uptake, alterations in SR Ca ATPase protein and mRNA levels, and correlation of the above changes with changes in hemodynamic indices during LVH progression must be conducted.

The studies reviewed here also do not clarify whether down-regulation of SERCA2 gene expression contributes to the transition from "compensated" hypertrophy to CHF. To date, there are no reliable "molecular markers" that can identify the transition from compensated hypertrophy to CHF, or guide therapy for this disorder. There is still a lack of convincing evidence to link the defect in SR Ca^{2+} uptake with hemodynamic abnormalities during the transition to CHF. Only very recently, Hasenfuss et al. (1994), reported that protein levels of SR Ca^{2+} ATPase were reduced in failing human myocardium. SR Ca^{2+} ATPase protein levels were closely related to SR Ca^{2+} uptake and more importantly, were also correlated to the force-frequency behavior of human myocardium. Therefore, their results suggest SR Ca^{2+} ATPase protein levels indeed determine the systolic contractile reserve with respect to frequency potentiation of contractile force in the human heart. Feldman et al. (1993) showed that a reduction in SERCA2 mRNA levels was linked to the progression of clinical and hemodynamic indices of LV systolic dysfunction in a rat model of LV pressure overload (thoracic AoC). However, *in vivo* hemodynamic measurements were not performed (due to the position of the constricting aortic band) so that the question of causality was not adequately addressed. Furthermore, pressure overload produced by abdominal AoC is an experimental model of LVH that seems to more closely mimic human chronic pressure-overload. This dissertation study will provide a comprehensive study of *in vivo* hemodynamics and changes in SERCA2 and contractile protein gene expression over time to determine whether alterations in SR Ca^{2+} pump activity contributes to the transition from LVH to CHF in the rat abdominal AoC model.

It is well known that the function of the cardiac SR Ca^{2+} pump is modulated by a phosphoprotein, namely phospholamban, with a molecular weight of 25 kDa (Kirchberger et al., 1974). Phosphorylation of phospholamban by cAMP-dependent protein kinase significantly increases the rate of Ca^{2+} uptake (Kranias et al., 1985). There are no isoforms of phospholamban, and the same protein is expressed in cardiac and slow-twitch skeletal muscle (Fujii et al., 1987). It has been reported that phospholamban mRNA levels are down-regulated in pressure overload hypertrophy and CHF in both animal models (Nagai, et al., 1989, Matsui, et al., 1995) and in humans (Feldman et al., 1991 and Arai et al., 1993). Interestingly, phospholamban mRNA shows a decrease parallel to that of SERCA2 in failing hearts, indicating that the expression of these two genes may be coordinately regulated.

TABLE 2

Alterations in SR Ca^{2+} uptake in pressure overload hypertrophy.

Animal Models	Functional Study		Protein Level	mRNA Study
	Ca^{2+} uptake	E-P	SR Ca ATPase	SERCA2
<u>Thoracic AoC</u> rat (Takahashi et al. 1992)				\leftrightarrow adult, \downarrow old
rat (Feldman et al., 1993)				\leftrightarrow 8, 20 wk, non-HF \downarrow 20 wk, HF
<u>Abdominal AoC</u> rat (Komuro et al., 1989)	\downarrow		\downarrow	\downarrow
rat (de la Bastie et al., 1990)	\downarrow	\downarrow	\leftrightarrow mild LVH \downarrow severe LVH	\leftrightarrow mild LVH \downarrow severe LVH
rat (Levitsky et al., 1991)	\downarrow	\downarrow	\downarrow	\downarrow
pig (Dhalla et al., 1984)	\uparrow 4 wk, \downarrow 8 wk			
rat (Limas et al., 1980)	\uparrow	\uparrow		
guinea pig (Cory, 1994)	\downarrow		\uparrow	
<u>PA banding</u> rabbit (Matsui, 1995)	\downarrow	\downarrow	\downarrow	\downarrow
<u>SHR</u> Cuneo & Genda, 1988	\uparrow			
Boluyt et al., 1994				\leftrightarrow

AoC, aortic coarctation; E-P indicates phosphorylated intermediate of the Ca^{2+} -ATPase; PA, pulmonary artery; SHR, spontaneously hypertensive rat; \uparrow , increase; \rightarrow , same as control; \downarrow , decrease. HF, heart failure.

c). *Cellular and molecular mechanisms involved in regulating cardiac SERCA2 gene expression.* To date, little is known about the cellular and molecular mechanisms involved in the regulation of SERCA2 gene expression. Thyroid hormone has been found to regulate the SERCA2 gene in cardiac myocytes both *in vivo* and *in vitro*. Exogenous administration of thyroid hormone increases cardiac SERCA2 mRNA and protein levels *in vivo* and in cell culture (Arai et al., 1991, Nagai et al., 1989, Rohrer et al 1988, 1991, Ojamaa et al., 1992, Zarain-Herzberg et al., 1994), whereas experimentally produced hypothyroidism decreases SERCA2 gene expression *in vivo* (Arai et al., 1991, Rohrer et al., 1988). In addition, fibroblast growth factors such as acidic and basic fibroblast growth factor (aFGF and bFGF) and transforming growth factor type β (TGF- β) have been found to down-regulate SERCA2 mRNA levels in cultured neonatal rat ventricular myocytes (Parker et al 1990), although the molecular mechanisms responsible for this down-regulation are not known.

Since the SERCA2 gene is tissue-specific, developmentally regulated, and responsive to specific pathophysiological conditions of heart muscle, it is an excellent model gene to study the complexity of myocyte transcriptional control. Transcription is a primary control point in the regulation of gene expression. The transcriptional machinery integrates regulatory information through the binding of *trans*-acting factors to *cis*-acting DNA elements. These DNA elements are found both proximal and distal to the transcription initiation site, and are involved in the activation or repression of transcription. Fisher et al (1993) and Rohrer et al (1991) have characterized upstream regulatory sequences from the transcriptional start site to -658 bp of rabbit SERCA2 gene and -559 bp of the rat SERCA2

gene respectively, as regions producing high-level transcriptional activity in cardiac myocytes. The 5'-flanking region of the SERCA2 gene is very G/C-rich. This region contains several consensus sequences that are likely to be important sites of interaction with nuclear proteins involved in regulating gene transcription. These include a "TATA box" like 5'-GATAAA-3' sequence, a "CAAT box" (5'-GCCAAT-3') sequence, and 2-3 SP1 consensus sequences (5'-GGCGGG-3') (Zarin-Herzberg et al., 1990, Sukovich, 1993, and Rohrer et al., 1991). These bind, respectively, the transcription factor II complex, the CTF family of transcription factors, and the zinc finger protein SP1, which have been previously shown to promote transcription of a variety of eukaryotic muscle and nonmuscle genes (Faisset and Meyer, 1992). A similar 17 bp upstream promoter element (UPE) with 88% homology in rabbit and rat was also identified in the proximal promoter region of SERCA2 gene (Sukovich, 1993, and Rohrer et al., 1991). Gel mobility shift and Southwestern analyses using the 17 bp UPE have revealed a specific DNA binding complex referred to as Ca^{2+} ATPase promoter factor-1 (CaPF1). The binding factor has an approximate M_r of 43 kDa and is a novel DNA-binding protein which plays a role in SERCA2 gene expression in skeletal muscle (Sukovich, 1993). One to three thyroid hormone-responsive elements (TREs) have also been identified within the 5' upstream regulatory sequences of the rat and rabbit SERCA2 genes respectively (Rohrer, 1991, Hartong et al., 1994 and Zarin-Herzberg et al., 1990, 1994). These sequences bind nuclear thyroid hormone receptors and up-regulate SERCA2 transcription in response to exogenous T3. Although it appears that other humoral signals in addition to thyroid hormones, and mechanical factors may be involved in

SERCA2 gene regulation, their effects on SERCA2 gene transcription, mRNA processing and stability, and translation are unknown.

E. CARDIAC MYOCYTE HYPERTROPHY --*IN VITRO* MODEL

The reduced SERCA2 mRNA levels observed in pressure-overloaded, hypertrophied hearts occurs in the presence of normal circulating levels of thyroid hormones, indicating that other factors (including a direct effect of mechanical load) may regulate SERCA2 gene expression *in vivo*. Obviously, it is difficult to study the role of mechanical factors in the regulation of SERCA2 gene expression during pressure overload, and to identify second messengers that may be involved in transducing mechanical signals from the cell surface to the nucleus. One critical advance in the study of the mechanisms of cardiac myocyte hypertrophy has been the development and characterization of primary cultures of neonatal rat myocytes (Chien et al., 1991).

1. Induction of Cardiac Myocyte Hypertrophy *in vitro*

Mechanical and hormonal factors have often been proposed as the initial triggers inducing the well-known qualitative changes in cardiac gene expression during pressure overload LVH. Pressure overload produces two different mechanical effects on the myocardium: increased passive stretch and increased active tension development. The effects of both mechanical stimuli on induction of cardiac myocyte hypertrophy have been

widely investigated using primary cultures of neonatal rat ventricular myocytes. It has been documented that either spontaneous (McDermott et al., 1985, 1989a and 1989b, Samarel and Engelmann, 1991), or electrically stimulated (McDonough and Glembotski, 1992, Johnson et al., 1994) contractile activity, and passive stretch (Komuro et al., 1991, Sadoshima et al., 1992) of cultured cells mimics many features of the pressure overload-induced hypertrophic response *in vivo*. On the other hand, diverse and distinct hormonal stimuli that may be important regulators of pressure overload hypertrophy *in vivo* have clear growth-promoting effects in the *in vitro* system. A number of studies have demonstrated that cardiac myocyte hypertrophy can be induced in cell culture by α_1 -adrenergic agonists (Simpson et al., 1991, Waspe et al., 1990), endothelin (Suzuki et al., 1990), angiotensin II (Ang II) (Baker and Aceto, 1990, Sadoshima and Izumo, 1993 a and b), peptide growth factors (e.g. transforming growth factor- β , fibroblast factors. Parker et al., 1990), serum stimulation (Qi et al., 1994), and α -thrombin receptor activation (Glembotski et al., 1993).

2. Characteristics of Cardiac Myocyte Hypertrophy *in vitro*

Cardiac myocyte hypertrophy induced by mechanical and hormonal stimuli *in vitro* have the following common features: (1) an increase in the size of myocytes accompanied by accumulation of myocyte protein (as evidenced by increases in total protein content, protein/DNA ratio and protein synthetic capacity); (2) the induction of IE response genes such as *c-fos*, *c-jun*, and *Egr-1*; (3) the re-expression of “fetal”, late response genes such as

ANF, MHC- β and α -skeletal actin; and (4) the up-regulation of the constitutively expressed contractile protein myosin light chain 2 (MLC-2) gene.

3. Mechanical Load Induced Cardiac Myocyte Hypertrophy.

In primary cultures of neonatal rat cardiac myocytes, spontaneous and electrically stimulated contractile activity produces myocyte hypertrophic growth, as measured by total protein and RNA accumulation (Allo et al., 1992, McDermott et al., 1989, Johnson et al., 1994 and Samarel and Engelmann, 1991). An increased protein synthetic capacity, as reflected by a net increase in rRNA content, is the primary translational mechanism by which the rate of protein synthesis is accelerated in spontaneously contracting or electrically stimulated cells (McDermott, 1991, Johnson et al., 1994). In terms of specific proteins, changes in the synthesis rates of actin and myosin have been shown to occur in contracting cells, either by transcriptional mechanisms involving selective changes in mRNA levels or by a post transcriptional process (Samarel and Engelmann, 1991 and McDermott et al., 1987). The present study also shows that contractile arrest up-regulates SERCA2 mRNA levels (Bassani et al., 1994). However, the signal transduction pathways involved in the gene regulation of contraction-induced cardiac myocyte growth is still largely unknown. Our previous studies (Samarel, Qi et al., 1994) have suggested that up-regulation of MHC- β may involve PKC activation. In contrast, McDonough and Glembotski (1992, 1994) found that electrically stimulated induction of ANF and MLC-2 gene expression was dependent

primarily on $[Ca^{2+}]_i$ and Ca^{2+} /calmodulin-dependent protein kinase II (CaMK) and independent of PKC.

The other widely used model to study how mechanical load induces the hypertrophic phenotype is to passively stretch cardiac myocytes that are adherent to an elastic membrane. Several studies (Komuro et al., 1990,1991, Yazaki and Komuro, 1992, Sadoshima et al., 1992) have demonstrated that stretching neonatal rat cardiac myocytes attached to laminin-coated silastic membranes induced myocyte hypertrophy as evidenced by an increase in protein synthesis and content without an increase in DNA synthesis. The phenotype of stretched cardiac myocytes *in vitro* highly resembles that of hemodynamic overload induced hypertrophy *in vivo*. Specifically, stretch causes a rapid and transient induction of a variety of IE genes (e.g. *c-fos*, *c-jun*, *c-myc*, JE and Egr-1), followed by activation of fetal genes, such as α -skeletal actin, MHC- β , and ANF which are all late response genes characteristic of rat ventricular hemodynamic overload *in vivo* (Komuro et al., 1990,1991, and Sadoshima et al., 1992). Stretch-induced *c-fos* gene expression is regulated at the transcriptional level, and the “stretch-responsive element” within the 5’ flanking region of the *c-fos* gene is identical to the previously described serum-response element (SRE) which binds serum response factor (SRF), a transcription factor that regulates the transcription rate of a variety of muscle and nonmuscle genes (Komuro et al., 1991, and Sadoshima et al., 1992). However, it appears that stretch-induced increases in MHC- β and ANF mRNAs may not be regulated at the transcriptional level (Sadoshima et al., 1992). These above mentioned effects could be prevented by pre-treatment of cells with the PKC inhibitors H7 and

staurosporine, or by prior down-regulation of PKC by chronic exposure to phorbol esters. Myocyte stretching results in small but significant increases in the levels of inositol phosphates, 1,2-diacylglycerol (DAG) and PKC activity, suggesting that PKC is activated by mechanical stress (Komuro et al., 1991, Yazaki et al., 1993, Sadoshima and Izumo, 1993 and Komuro and Yazaki, 1994). Myocyte stretching was also shown to activate mitogen-activated protein (MAP) kinase (Yamazaki et al., 1993 and Sadoshima and Izumo, 1993). When PKC was depleted by preincubating myocytes with phorbol ester or its activity was blocked by the PKC inhibitor staurosporine, stretch-induced MAP kinase activity was decreased by 60-70%. These results suggest that the stretch-induced increases in MAP kinase activity occurs through both PKC-dependent and independent pathways. Subsequently, Sadoshima and Izumo (1993) demonstrated that myocyte stretching causes rapid activation of multiple second messenger systems, which may in turn initiate a cascade of pleiotropic responses. Unfortunately, little is known as to how muscle cells sense mechanical load and transduce it into intracellular signals of gene regulation (Sadoshima et al., 1992).

4. Hormone Induced Myocyte Hypertrophy.

Angiotensin II (Ang II) acts as a direct growth factor for cardiac myocytes. It causes a rapid induction of IE genes such as *c-fos*, *c-jun*, and *Egr-1* and hypertrophy in cardiac myocytes (Baker and Aceto, 1990, Sadoshima and Izumo, 1993b & c). Ang II also induces the expression of “fetal” genes such as ANF, α -skeletal actin and up-regulates the

expression of growth factor genes, such as TGF- β and angiotensinogen (Sadoshima and Izumo, 1993b). Sadoshima and Izumo (1993c) also found that Ang II activated multiple phospholipid-derived second-messenger systems. PLC and PKC seem essential for Ang II-induced *c-fos* gene expression, whereas Ca^{2+} may play a permissive role. The “Ang II response element” of the *c-fos* promoter also maps to the PKC-dependent portion of the serum response element.

Chronic exposure to an α_1 -adrenergic agonist causes hypertrophic growth of cultured neonatal rat ventricular myocytes. This hypertrophic response is accompanied by activation of PKC activity, which can be inhibited by incubating myocytes with the PKC inhibitor staurosporine (Henrich and Simpson, 1988, Allo et al., 1992). These results indicate that PKC activation may play a very important role in α_1 -adrenergic activation of cardiac myocyte hypertrophy.

F. ROLE OF PKC ACTIVATION IN CARDIAC MYOCYTE HYPERTROPHY AND GENE EXPRESSION

As is apparent from the information presented above, protein kinase C activation may be centrally important in signaling the hypertrophic phenotype in response to a diverse number of exogenous stimuli. Therefore, a brief overview of protein kinase C structure and function in cardiac tissue is now presented.

1. PKC Isoenzymes in Heart.

a). PKC isoforms. PKC is a family of closely related serine-threonine protein kinases that can be classified into conventional PKCs (cPKC), new PKCs (nPKC) and atypical PKCs (aPKC). As shown on Table 3, there are 10 identified subspecies of PKC in mammalian tissues (Asaoka et al., 1992). The cPKCs are characterized enzymatically by their requirement for Ca^{2+} , phospholipids, and DAG or phorbol ester for activation. This reflects the presence of four conserved domains. The C1 region is the putative phospholipid-and DAG/phorbol ester-binding domain, the C2 region is the putative calcium-binding domain, and the C3 and C4 regions constitute the kinase domain. The nPKCs are structurally related to the cPKCs. However, they lack the C2 domain and do not require Ca^{2+} for maximal activation. PKC ζ also differs from other members of the PKC gene family in that it contains only one cysteine-rich zinc finger like motif in the C1 domain. As a result, neither Ca^{2+} nor DAG/phorbol ester activates this PKC isoform (Figure. 2) (Nishizuka, 1992).

TABLE 3

PKC subspecies in mammalian tissues

Group	Subspecies	Molecular mass (kDa)	Activators ^a	Tissue expression
cPKC	α	76,799		Universal
	β I	76,790	Ca^{2+} , DAG,	Some tissues
	β II	76933	PS, FFA,	Many tissues
	γ	78366	LysoPC	Brain only
nPKC	δ	77517	DAG, PS	Universal
	ϵ	83474	DAG, PS, FFA	Brain and other tissues
	η (L)	77972	?	Lung, skin, heart
	θ	81571	?	Skeletal muscle
aPKC	ζ	67740	PS, FFA	Universal
	λ	67200	?	Ovary, testis and others

a. The activators for each subspecies are determined with calf thymus H1 histone and bovine myelin basic protein as model phosphate acceptors.

Abbreviations: DAG, diacylglycerol; PS, phosphatidylserine; FFA, *cis*-unsaturated fatty acid; LysoPC, lysophosphatidylcholine.

Adapted from Asaoka, et al., 1992.

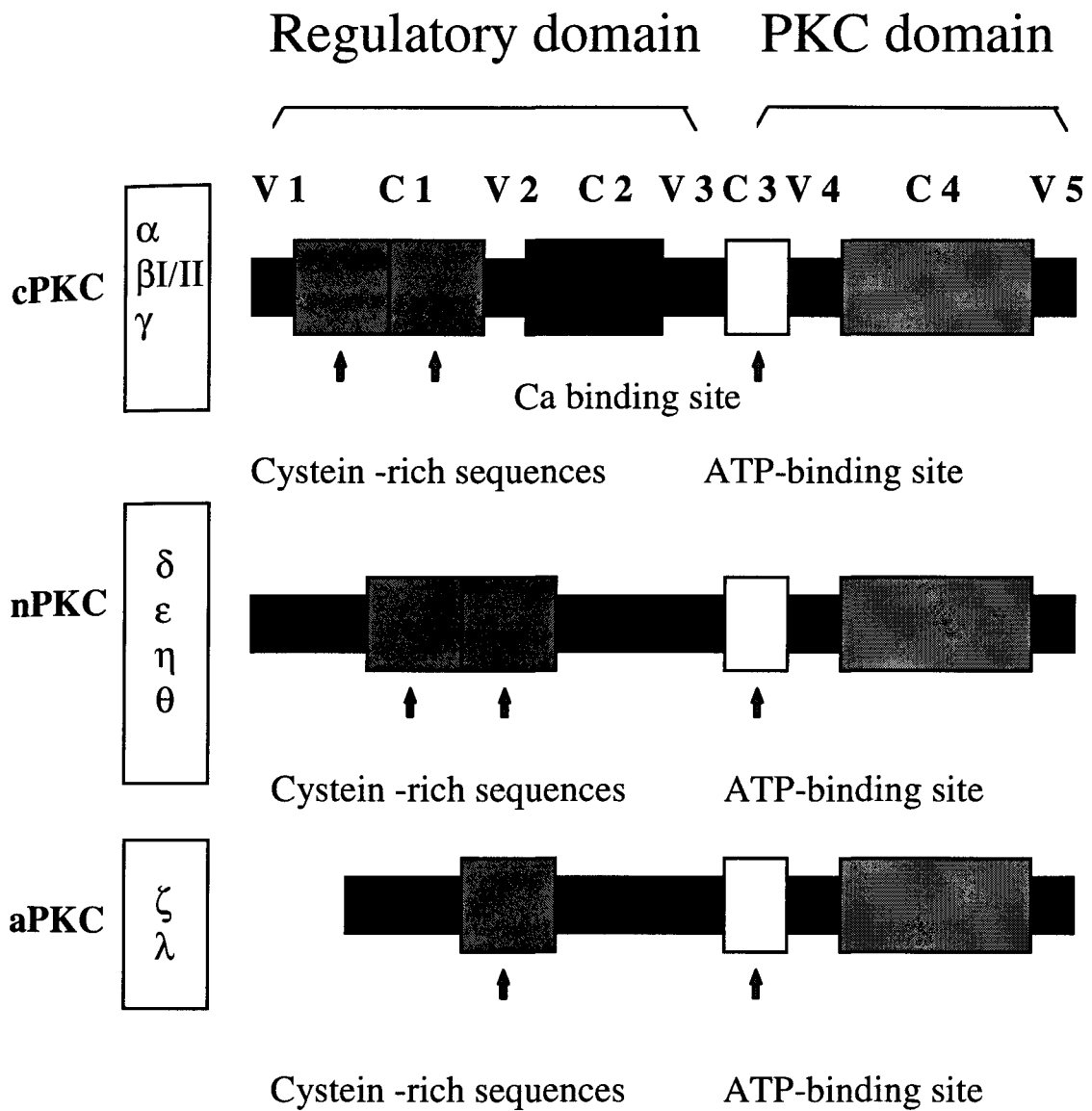


Figure 2: Structure of PKC subspecies. C = constant. V = variable.

b). Activation and down-regulation of different PKC isoenzymes in heart. Currently, there is some controversy concerning which PKC isoforms are expressed in adult and neonatal rat cardiac myocytes and which PKC isoenzymes mediate cardiac myocyte functions. Bogoyevitch et al (1993) reported that only ϵ -PKC was expressed in adult rat cardiac myocytes. However, RT-PCR, along with restriction enzyme mapping and DNA sequencing revealed the presence of α -, δ -, ϵ -, θ - and ζ -PKC isoforms in adult rat cardiac myocytes and cultured neonatal ventricular myocytes (Kohout and Rogers, 1993). A recent publication by Puceat et al (1994) using Western blot analysis reported that both neonatal and adult rat cardiac myocytes express α -, δ -, ϵ -, and ζ -PKC isoenzymes. Furthermore, Rybin and Steinberg (1994) found different patterns of PKC isoenzyme expression in neonatal (α -, δ -, ϵ -, and ζ -PKC) and adult rat (δ - and ϵ -PKC) cardiac myocytes. Church et al (1993) demonstrated that neonatal cardiac myocytes contain α -, β -, δ -, and ζ -PKC but not ϵ -PKC isoenzymes by Western blotting analysis. Mochely-Rosen and coworkers (Mochely-Rosen et al., 1990, Disatnik et al., 1994) showed by immunofluorescence studies the presence of α -, β I-, β II-, δ -, ϵ - and ζ -PKC isoenzymes in neonatal cardiac myocytes, each of which is translocated to distinct subcellular sites after stimulation by norepinephrine or PMA. Differences in translocation site of individual isoenzymes- and, potentially, their phosphorylation of different substrates localized at these sites may explain the diverse biological effects of PKC. A recent study (Gu and Bishop, 1994) found that α -, β I-, β II-, ϵ -, and ζ -PKC isoenzymes were present in both soluble and particulate fractions, whereas PKC δ was only present in the nuclear-cytoskeleton of the particulate fraction of normal adult rat

heart tissue. PKC- β I, β II and PKC- ϵ were increased in response to pressure overload (Gu and Bishop, 1994). These results further suggest that PKC plays an important role in the regulation of cardiac myocyte growth and function in the development of hypertrophy and that individual isoenzymes may perform different functions in response to pathological conditions.

There have also been several studies of PKC isoenzyme translocation and down-regulation in cardiac myocytes (Disatnik et al., 1994, Bogoyevitch et al., 1993 and 1994, Puceat et al., 1994, Rybin and Steinberg et al., 1994 and Church et al., 1993). Puceat et al. (1994) found that α_1 -adrenergic or P_2 purinergic receptor activation in neonatal cardiac myocytes elevated membrane-associated immunoreactivity of δ - and ϵ -PKC. In addition, they found that a short treatment with PMA (100 mM) caused redistribution of α -, δ -, and ϵ -PKC isoenzymes in these cells. Longer PMA treatment caused substantial down-regulation of the α - and δ -PKC but not the ϵ -PKC isoenzyme. These authors also found a PKC-mediated increase in myristoylated alanine-rich PKC substrate (MARCKS) phosphorylation and *c-fos* mRNA accumulation. The former but not the latter function was correlated with the persistence of ϵ -PKC after PKC activation and down-regulation. Johnson and Mochly-Rosen(1995) used many different antisera to demonstrate that PMA has differential effects on the distribution and levels of α , β I, β II, δ , ϵ and ζ -PKC isoenzymes in cultured neonatal rat ventricular myocytes. In agreement with Puceat et al., their study found α -PKC to be the most sensitive and ϵ -PKC to be the least sensitive PKC isoenzyme to PMA-induced down-regulation. However, in contrast to Puceat et al., they found the δ -PKC isoenzyme was

similarly resistant to PMA induced down-regulation as ϵ -PKC. The above mentioned study also suggested that the ϵ -PKC isoenzyme may modulate the contraction rate of neonatal cardiac myocytes.

2. PKC Activity in Cardiac Hypertrophy *in vivo*

Several types of cardiac hypertrophy have been associated with changes in PKC activity in cardiac tissue. Both cytosolic and particulate PKC activities were to be found markedly increased in cardiac tissue of hypertrophic myopathic hamsters (Chambers and Eilon, 1988, Chi et al., 1993). In the rat with thoracic AoC, cytosolic and membrane associated PKC activities were significantly elevated. The increased PKC activity may be involved in the increased phosphorylation of cardiac MLC protein in myocardial hypertrophy (Kwiatkowska-Patzer and Domanska-Janik, 1991). It has been reported that particulate PKC activity was enhanced in stroke-prone spontaneously hypertensive rat heart cells in response to norepinephrine stimulation (Kawaguchi et al., 1993). A recent study demonstrated that PKC activity and content were increased during development of pressure overload induced LVH. Furthermore, the increased PKC isoenzymes were mainly limited to PKC_{1,2} and PKC- ϵ , and the increases were present mainly in the membrane and nuclear-cytoskeletal fractions (Gu and Bishop, 1994).

3. Role of PKC Activation in Cardiac Myocyte Hypertrophy *in vitro*

As mentioned previously, PKC activation has been associated with mechanically (spontaneous contraction or passive stretch) and hormonally (Ang II and α_1 -adrenergic activation) induced cardiac myocyte hypertrophy *in vitro*. PKC activation by PMA in cell culture also reproduces many of the changes in gene expression that occur in the pressure overloaded heart *in vivo*, such as an increase in protein synthetic rate (Fuller and Sugden, 1989), induction of IE gene expression (Dunnmon et al., 1990) and up-regulation of MLC-2 (Shubeitha et al., 1992), ANF (Shubeitha et al., 1992), α -skeletal actin (Komuro et al., 1991) and MHC- β (Kariya et al., 1991, Samarel and Engelmann, 1991). Co-transfection of MLC2 and MHC- β promoter-reporter gene constructs with constitutively active, mutant forms of PKC (Shubeita et al., 199, Kariya et al., 1991, respectively), and nuclear run-on assays of PMA-treated neonatal rat myocytes (Dunnmon et al., 1990) demonstrated that the hypertrophic effects of PKC activation are in part regulated at the level of gene transcription. However, phorbol esters may also stimulate rat myocyte protein synthesis at the translational level by increasing protein synthetic efficiency (Fuller and Sugden, 1989) and capacity as evidenced by an increase in total RNA and rRNA (Allo et al., 1991, 1992). In contrast, inhibition of PKC activity by the protein kinase C inhibitor staurosporine in contracting cells markedly decreased MHC- β mRNA levels (Qi et al., 1994, Samarel and Engelmann, 1991). Overall, these results suggest that PKC activation may play an important role in many of the signal transduction pathways leading to cardiac myocyte hypertrophy. However, very little is known about the role of PKC in the regulation of

SERCA2 gene expression in these cell culture models. This will be specifically addressed in this thesis.

G. ROLE OF $[Ca^{2+}]_i$ IN CARDIAC MYOCYTE HYPERTROPHY.

Since changes in $[Ca^{2+}]_i$ accompany alterations in the contractile state of the myocardium and elevation of $[Ca^{2+}]_i$ can activate several isoforms of PKC, it has long been thought that alterations in $[Ca^{2+}]_i$ might represent a logical stimulus for coupling changes in contractility with alterations in cardiac metabolism and gene expression in response to pressure overload (Kaibuchi et al., 1986, MacKinnon et al., 1988). Although no direct role for the changes in $[Ca^{2+}]_i$ during cardiac hypertrophy have yet to be described, inhibition of spontaneous contractile activity by Ca channel blockers decreases the capacity for protein synthesis, inhibits total myosin synthesis and accumulation, and markedly reduces MHC- β gene expression in neonatal rat heart cells (Qi et al., 1994, Samarel and Engelmann, 1991). In addition, the down-regulation of the SERCA2 gene can alter $[Ca^{2+}]_i$ by slowing Ca re-uptake by the SR. It is possible that changes in $[Ca^{2+}]_i$ may regulate SERCA2 gene expression via a negative feed-back loop. Therefore, the role that $[Ca^{2+}]_i$ plays in regulating SERCA2 gene expression will be specifically investigated in the present study.

H. SUMMARY.

Pressure overload LVH and CHF are often associated with impaired diastolic function. $[Ca^{2+}]_i$ during relaxation which is primarily controlled by Ca^{2+} uptake via the SR Ca^{2+} ATPase. It has been well documented that SR Ca^{2+} ATPase mRNA and protein levels as well as SR Ca^{2+} uptake are decreased during pressure overload hypertrophy and CHF in experimental animals and humans. Clearly, a potential cause for slowed relaxation may be related to the activity of SR Ca^{2+} ATPase. However, there is still no evidence to show a direct “cause and effect” relationship between the down-regulation of the SERCA2 gene and impaired diastolic function during pressure overload hypertrophy and CHF. More importantly, the studies reviewed here do not clarify whether down-regulation of SERCA2 gene expression contributes to the transition from “compensated” hypertrophy to CHF. Therefore, this dissertation study will provide a comprehensive study of *in vivo* hemodynamics and changes in and SERCA2 and compositional proteins (including myosin isoenzymes and fibrillar collagens) during the progression of rat pressure overload LVH to determine whether alterations in SR Ca^{2+} ATPase contributes to the transition from LVH to CHF.

Although thyroid hormone is known to regulate the SERCA2 gene at the transcriptional level, the cellular and molecular mechanisms responsible for the regulation of SERCA2 gene expression during pressure overload hypertrophy and CHF are still largely unknown. Mechanical stimuli are known to play a very important role in the induction of LVH in the

pressure overloaded heart. PKC and other kinases are likely to be involved in transducing extracellular stimuli during pressure overload hypertrophy. Whether mechanical stimuli and PKC activation are involved in the regulation of SERCA2 gene expression in pressure overloaded and failing hearts are still unknown but will be thoroughly studied in this dissertation work using primary cultures of neonatal rat ventricular myocytes.

CHAPTER III

MATERIALS AND METHODS

A. REAGENTS.

1. Materials and Culture Media for Cell Culture Experiments

Tissue culture plates were obtained from Costar (Cambridge, MA). Swim's-S77, Ca^{2+} - Mg^{2+} -free Hanks' balanced salt solution (HBSS), Antibiotic-Antimycotic Solution (100 \times), Gentamycin, L-glutamine, Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (DMEM/F12), Acid soluble collagen (Calf skin type I), actinomycin D (Act-D), (\pm)-verapamil and 4α -phorbol were purchased from Sigma Chemical Co (St. Louis, MO). Staurosporine and chelerythrine were obtained from LC Laboratories. (Woburn, MA). Collagenase (type CLSII) was obtained from Worthington Biochemical Corp. (Freehold, NJ). Fetal bovine serum was purchased from HyClone Laboratories, Inc.(Logan, Utah). Lipofectamine and Optimem I medium were obtained from GIBCO-BRL (Cleveland, OH). All other reagents were of the highest grade commercially available and were obtained from Sigma and Baxter S/P (McGaw Park, IL).

PC-1 medium: PC-1 tissue culture medium was obtained from Hycor Biomedical Inc.(Irvine, CA). According to the manufacturer, PC-1 medium is formulated in a specially modified DMEM/F12 base and contains a complete HEPES buffering system with insulin (15 $\mu\text{g/ml}$), T_3 (0.5 nM), fatty acids, and proprietary proteins (at a concentration of less than 530 $\mu\text{g/ml}$). 500 ml of PC-1 medium was completed by adding 1 vial of PC-1 supplement, 5 ml of 2 mM L-glutamine, and 5 ml of Antibiotic-Antimycotic Solution (100 \times).

2. Reagents for Molecular Biology Assays.

Agarose was obtained from FMC BioProducts (Rockland, ME). Guanidine thiocyanate was purchased from Fisher Biotech (Fair Lawn, NJ). 3-[N-Morpholino] propane sulfonic acid (MOPS) was obtained from Sigma. $20 \times \text{SSC}$ (3 M NaCl and 0.3 M Na Citrate, pH 7.0), salmon sperm DNA, 50% Dextran sulfate were purchased from 5 Prime-3 Prime Inc (Boulder, CO). Restriction enzymes and Taq polymerase were obtained from Phamacia Biotech (Piscataway, NJ). Competent *E. Coli* 5H α (DH5 α), T_4 polynucleotide kinase, T_4 DNA ligase, deionized formamide, Luria-Bertani (LB) medium, 123 bp and 1 kb DNA ladders were obtained from GIBCO-BRL. Multiprime DNA labeling system, (α - ^{32}P)dCTP, (α - ^{35}S)dATP, and (γ - ^{32}P)ATP were purchased from Amersham. QIAGEN plasmid purification kit was purchased from QIAGEN INC. (Chatsworth, CA). Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH, clone pHcGAP) cDNA was obtained from American Type Culture Collection (Rockville, MD) and the rat SERCA2

cDNA clone is a gift from Dr. Wolfgang Dillmann, (UCSD). 40-base oligonucleotide probes specific for the 3' untranslated regions of the rat MHC- α and MHC- β genes were obtained from Oncogene Science, Uniondale, NY. An oligonucleotide probe specific for rat 18S rRNA (5'-ACGGTATCTG ATCGTCTTCG AACC-3') (Chan et al., 1984) was kindly synthesized by Dr. Bassam Wakim (Loyola University Medical Center, Maywood, IL). Sequenase version 2.0 DNA sequencing kit was obtained from USB (Cleveland, OH).

3. Reagents for Biochemical Assays

Rabbit anti-rat SERCA2 antiserum was a generous gift from Drs. Ronald Hartong and Wolfgang Dillmann, UCSD. Goat anti-rabbit IgG conjugated with horseradish peroxidase was purchased from CAPPEL Inc (Durham, NC). Protein rainbow molecular weight standards and enhanced Chemoluminescence kits were obtained from Amersham.

B. ADULT RAT ABDOMINAL AoC MODEL.

Male Sprague-Dawley rats (Harlan Industries, Indianapolis, IN) weighing 160-190 g were randomly divided into sham and aortic banded groups. Animals were anesthetized with an intramuscular injection of ketamine (70 mg/kg) and xylazine (2 mg/kg). A midline abdominal incision was used to expose the left renal vascular bundle and the

abdominal aorta was dissected free to isolate a 2-cm segment. Coarctation of the abdominal aorta was produced using a tantalum hemoclip as previously described (Eleftheriades et al., 1993). The applicator was modified to allow passage of a 25 g needle through the hemoclip when completely closed. Sham operated animals underwent anesthesia and dissection of the abdominal aorta without application of the suprarenal band. The peritoneum was closed with 5-0 silk suture and the skin was closed with a 9 mm Autoclip applicator (Clay Adams, Parsippany, NJ). All animals received food and water ad libitum. Sham and banded animals were investigated at 8 and 16 weeks after surgery.

C. HEMODYNAMIC MEASUREMENTS *IN VIVO*.

Rats were anesthetized with ketamine-xylazine, body weight was recorded, and the right carotid artery was exposed through a small cervical incision. A 3-F micromanometer-tipped catheter (MMI-Gaeltec, Hackensack, NJ) interfaced to a Gould 2400S Recorder (Gould Instrument Systems, Valley View, OH) was inserted into the right carotid artery and advanced to the level of the aortic arch. After recording systolic, diastolic and mean arterial pressures (SAP, DAP, and MAP, respectively), the catheter was advanced across the aortic valve to obtain LV systolic and diastolic pressures. Analog signals were simultaneously digitized (1,000 Hz) and stored on videotape for

subsequent analysis of peak systolic LV pressure (LVP), LV end-diastolic pressure (LVEDP), maximal $+dP/dt$ ($+dP/dt_{\max}$, defined as maximal rate of rise of the LVP), maximal $-dP/dt$ ($-dP/dt_{\max}$, defined as maximal rate of fall of the LVP), contractility index (CI, defined as $(+dP/dt_{\max})/LVP$ at peak $+dP/dt$), and the time constant for isovolumic relaxation (τ) using the PO-NE-MAH algorithm software package. τ was computed using the linear regression of dP/dt versus LVP during isovolumic relaxation as described by Martin et al. (1984). Animals were then administered an overdose of pentobarbital and the heart was excised and quickly weighed. After further dissection, the left ventricle (LV free wall + septum) was weighed, immediately frozen in liquid N_2 and stored at $-70^\circ C$ for further analysis.

In some experiments, Ca^{2+} tolerant, LV myocytes were isolated from the hearts of sham and 16 wk banded rats as previously described (Bassani et al., 1992). Freshly isolated cells were pelleted by centrifugation, resuspended in 150 mM NaCl, and homogenized.

D. HYDROXYPROLINE ANALYSIS.

LV tissue (100-200 mg) was homogenized in 19 vol of 150 mM NaCl. After removal of an aliquot for analysis of total protein content by the Lowry method, a portion was added to 2 vol of ethanol, and the precipitated proteins were hydrolyzed in 6N HCl ($110^\circ C$, 24 h). The hydrolysates were evaporated to dryness, reconstituted in water, and

clarified by treatment with activated charcoal followed by centrifugation (3000 g, 20 min.). The hydrolysates were then assayed for hydroxyproline concentration by the spectrophotometric method of Woessner (1961). Data were expressed as nmol hydroxyproline per g wet weight tissue.

E. ISOLATION AND PRIMARY CULTURE OF NEONATAL RAT VENTRICULAR MYOCYTES.

1. Coating Cell Culture Plates.

Collagen Stock Solution (20×): Acid soluble collagen was dissolved in 1:10 diluted Swim's-S77 (pH 4.2) and stirred overnight at 4 °C until it was completely dissolved. The final concentration was adjusted to stock solution 0.5 mg/ml and stored at -20 °C in aliquots.

Cell culture dishes were pre-treated with collagen (Engelmann and Fierer, 1982). The collagen stock solution was thawed and diluted with Swim's-S77 to a final concentration to 25 µg/ml. A recommended amount of collagen solution (1.5 ml for 35 mm dish or each well of a 6 well plate, 6 ml for 100 mm dish) was added onto the plate. The plate was swirled to cover the bottom and left at 4° C overnight. The remaining solution was aspirated, fresh Swim's-S77 solution was added, and the plates were stored at 4° C.

2. Cell Isolation Protocol.

Neonatal ventricular myocytes were isolated from the hearts of 2-day-old rats by the collagenase digestion method (Engelmann et al., 1990). Neonatal rat pups, at postnatal day 1 to 2, were decapitated into liquid nitrogen, bodies washed with 70% ethanol, and the chest cavity opened to expose the heart. The beating hearts were rapidly removed and placed into ice-cold, CO₂-equilibrated perfusion buffer without collagenase and Ca²⁺ (Table 4). Hearts were collected and ventricles were trimmed free of atria and major blood vessels, washed in fresh 37° C perfusion buffer and minced with an alcohol-cleaned new razor blade. About 6-10 hearts were placed into each 25 ml Erlenmeyer flask containing 5 ml of enzyme/calcium-free perfusion buffer and 5 ml of perfusion buffer with collagenase and calcium for the first 15 min incubation. The perfusion buffer containing 0.1 mM CaCl₂ was pre-warmed to 37° C overnight in a 5 % CO₂ tissue culture incubator and the next morning, 0.5 mg of collagenase/ml was added to make enzyme digestion perfusion buffer.

Tissue fragments were shaken at 100 to 125 rpm for 15 to 23-minute incubations at 37° C. Cells, cell debris, and residual red blood cells that were released during the first 15-minute incubation were aspirated off and discarded. Another 8-10 ml of enzyme-containing perfusion buffer was added, tissue fragments were incubated for 18 min, and again released cells/tissue debris were discarded. Fresh enzyme digestion buffer (8-10 ml) was added, and incubated for 15 min. Then tissue fragments were triturated several times with a sterile, large-bore plastic transfer pipette, and incubated for an additional 7

min. After 22 min of digestion, tissue fragments were allowed to settle by gravity for 0.5 min, and all released cells were collected from the supernatant using a transfer pipette. Released cells were filtered through a sterile 40 mm nylon mesh that was washed with 3 ml of enzyme free buffer. Fresh enzyme containing buffer (8-10 ml) was added to the tissue fragments and this step was repeated two more times.

The cells released after each dissociation were isolated by centrifugation ($50 \times g$, 4 min, RT), and resuspended in 5 ml enzyme free perfusion buffer. The resuspension was added to a 50 ml collection tube containing 12.5 ml perfusion buffer, 90 μ l fetal bovine serum and 125 μ l of 0.5 M EDTA and stored on ice. At the second collection, another 125 μ l of 0.5 M EDTA was added to the collection tube. After the final collection, the volume was brought to 30 ml and total cell yield was determined using a hemocytometer. All released cells were collected by centrifugation, and resuspended in PC-1 medium.

3. Cell Culture

Released cells were plated onto collagen-coated dishes in undiluted PC-1 medium and left undisturbed in a 5% CO₂ incubator for 18 h. Unattached cells were then removed by aspiration. The cell layer was gently washed with HBSS. Growth medium (a 2:1 mixture of DMEM/F12:PC-1) was added. Verapamil, PMA or other agents were added at this time. Myocytes were then maintained in serum-free culture for up to 96 h with daily medium replacement.

TABLE 4

Perfusion Buffer For Neonatal Rat Ventricular Myocyte Isolation.

COMPONENT	AMOUNT (mg/l)	CONCENTRATION
HEPES	2,400	10 mM
Pyruvate, Na-Salt	1,100	10 mM
L-Glutamine	730	5 mM
Nicotinamide	120	1 mM
L-Ascorbate	70	0.4 mM
Adenosine	270	1 mM
D-Ribose	150	1 mM
MgCl ₂	240	1 mM
Taurine	375	1 mM
DL-Carnitine	400	2 mM
Potassium Bicarbonate	2,600	26 mM
Joklik MEM	1 package	
Gentamycin	1 ml (1000×)	10 µg/ml
Final pH	7.3-7.4	

This buffer was made up with 0.5 mg collagenase/ml and 0.1 mM CaCl₂ when used for cell isolation and tissue digestion.

F. TOTAL RNA ISOLATION FROM RAT LV TISSUES AND CULTURED NEONATAL MYOCYTES.

Total RNA was isolated from LV tissue and cultured neonatal myocytes by the method of Chomczynski and Sacchi (1987). For tissue samples, approximately 50 mg of frozen LV tissues were homogenized in 1 ml of Guanidine thiocyanate solution (GIT= 4 M Guanidine thiocyanate, 25 mM Na citrate, 0.5% Sarcosyl and 0.1 M β -mercaptoethanol) in a polypropylene tube for 30 sec. and made up to a final volume of 3 ml by adding another 2 ml GIT solution to the tube. For cultured cells, 100 mm dishes were washed with cold HBSS twice, scraped in 3 ml GIT solution. Then, 300 μ l of 2 M NaOAc (pH 4.0), 3 ml of phenol and 600 μ l of chloroform:isoamyl alcohol (49:1) were added to the tube with mixing after each addition, incubated on ice for 15 min, centrifuged at $10,000 \times g$ for 20 min at 4° C. The aqueous (top layer) phase was mixed with an equal volume of isopropyl alcohol to precipitate nucleic acids at -20° C overnight. The RNA was pelleted by centrifugation at $10,000 \times g$ as before, and resuspended in 400 μ l of GIT solution. The RNA was precipitated again by mixing with 600 μ l isopropyl alcohol, pelleted by centrifugation, washed with 70% ethanol and dried in a Speed Vac concentrator. The final RNA pellet was re-dissolved in 1 mM EDTA and stored at -70° C for Northern blot analysis.

G. SPECTROPHOTOMETRIC QUANTIFICATION OF DNA OR RNA

A small aliquot of the DNA or RNA sample was quantified by UV spectrophotometry. The readings at wavelengths of 260 nm and 280 nm were taken. An OD reading of 1.0 at 260 nm corresponding to approximately 50 µg/ml of double stranded DNA, or 40 µg/ml single stranded RNA. The purity of DNA and RNA preparations was estimated by the ratio of the readings at 260 and 280 nm (OD_{260}/OD_{280}). Pure preparations of DNA and RNA have OD_{260}/OD_{280} ratios between 1.8 and 2.0.

H. AGAROSE GEL ELECTROPHORESIS

1. DNA Agarose Gel Electrophoresis

DNA from 500 bp to approximately 10 kb in length can be separated on agarose gels of various concentrations (0.8-1.2%, w/v). Agarose was dissolved in 1× TAE buffer (50× TAE buffer (1 L): 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA, pH=8) by boiling, and cooled to 55° C. Ethidium bromide (EB) was added to a final concentration of 0.5 µg/ml and mixed thoroughly before casting the agarose solution in an appropriate size gel apparatus. The gel was allowed to set completely at room temperature, and depending on the agarose concentration, complete solidification

occurred in 30 to 60 min. The solidified gel was submerged in 1× TAE buffer until electrophoresis.

DNA samples were mixed with 6× loading buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol FF and 40 % (w/v) sucrose in H₂O) and loaded into the wells of the submerged gel. One or more DNA molecular weight standards were also included in each gel. The gel was electrophoresed at 1-5 volts/cm. The DNA bands were viewed under UV light and photographs were taken by using a photographic transilluminator system.

2. RNA Agarose Gel Electrophoresis

Agarose gels (1.2%, w/v) were prepared in the same way as the gels used for DNA electrophoresis except that agarose was dissolved in 1× MOPS solution (10× MOPS solution: 0.2 M MOPS, 0.05 M sodium acetate, 0.01 M EDTA, pH 7.0). After cooling to 55° C, 37% of formaldehyde (final concentration = 5%, v/v) and EB were added to the solution.

For Northern blotting, RNA samples (10 µg) were denatured in 1× MOPS, 5% formaldehyde and RNA loading dye (6× loading dye: 50 % glycerol, 1 mM EDTA (pH=8.0), 0.25 % bromophenol blue) at 65° C for 10 min. The denatured RNA samples were chilled on ice and centrifuged for 3 sec to deposit all of the fluid in the bottom of the microfuge tube. The RNA samples were loaded into the preformed wells. The gel was run at 100 volts for 1-2 h until the dye front reach to the end of the gel. RNA was

quantified by absorbance at 260 nm and the integrity was determined by examining the 28S and 18S rRNA bands in ethidium bromide-stained agarose gels visualized under UV light.

I. NORTHERN BLOTTING.

Following electrophoresis of RNA, the agarose gels were prepared for transfer by soaking in denaturing solution (0.15 M NaCl, 0.05 M NaOH) for 45 min followed by neutralizing in 0.15 M NaCl, 0.1 M Tris-HCl solution for 30 min. The RNA was transferred to nylon membranes (Micron Separations Inc., Westboro, MA) by capillary action with 10× SSC overnight. After transfer, the nucleic acids were cross-linked to the membrane using an UV cross-linker (Stratagene).

The membranes were hybridized with cDNA and oligonucleotides probes as follows. The RNA blots were prehybridized for several hours at 42° C in cDNA hybridization solution (50 % deionized formamide, 10 % Dextran sulfate, 1 % SDS, 1 M NaCl, and 100 µg/ml salmon sperm DNA (heat denatured by boiling 5 min)) and then hybridized overnight at 42° C with labeled probe in the same solution. After hybridization, the membranes were washed initially with 2× SSC and 0.5 % SDS at room temperature for 10 min (4 times). The final washes were in 0.1× SSC and 0.1 % SDS at 60° C. All blots were exposed to MP film (Amersham) with intensifier screens at -70° C for varying time periods. Band intensity was quantified using an LKB Ultrascan XL Enhanced Laser

Densitometer interfaced to a personal computer running Gelscan XL (Ver. 1.21). Band intensity of the SERCA2, MHC- α and MHC- β signals were expressed relative to the intensity of the constitutively expressed gene GAPDH.

J. FILTER STRIPPING AND REPROBING.

In order to reprobe the blots with several cDNA or oligonucleotide- probes, the hybridized blots were stripped by incubating in 0.5 % SDS at 85° C for 45 min. The blot can be reprobbed several times after stripping.

K. PREPARATION OF cDNA PROBES:

1. Transformation of Plasmid DNA into Bacteria.

To obtain large quantities of plasmid DNA, *E. Coli* bacteria were transformed by plasmid and allowed to multiply. DH5 α bacteria were removed from -80° C and thawed on ice for 15 min. 100 μ l of competent cells were placed into a chilled sterile polypropylene culture tube. 100 ng of plasmid DNA dissolved in 1 μ l TE buffer was gently added onto thawed bacteria. After incubating on ice for 30 min, the cells were heat shocked at 42 °C for 45 sec, and again chilled on ice for 2 min. Then 0.9 ml sterile LB broth and 2 μ l of 25 mg/ml ampicillin were added into the culture tube. The bacteria

were allowed to grow at 37° C for 1 h in a shaking incubator (225 rpm). Finally, the bacteria were spread out on an ampicillin agar plate and incubated in a 37° C incubator for 14-16 h to select for transformed bacteria.

2. Plasmid DNA Isolation

a). Small Scale Preparation of Plasmid DNA. To isolate small amounts of plasmid DNA from transformed bacteria, a rapid boiling method for the preparation of bacterial plasmids was employed (Holmes et al., 1981). A single bacterial colony was transferred into 5 ml of LB medium containing the appropriate antibiotic in a loosely capped 15 ml tube. The culture was incubated overnight at 37° C. A small volume of bacteria (0.5 ml) was stored at 4° C for large scale preparation of plasmid DNA. The rest of the bacteria were pelleted at maximum speed in a microcentrifuge for 30 s, and the supernatant was aspirated. The pellet was resuspended in 50 µl of lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1 mg/ml bovine serum albumin, 15 % wt/vol. sucrose, 2 mg/ml lysozyme and 0.2 mg/ml pancreatic RNase) and incubated at 37° C in a shaker for 5 min. The suspension was boiled for 60 sec and immediately chilled on ice for 60 sec. After centrifugation at maximum speed for 20 min at 4° C in a microcentrifuge, the supernatant was transferred to a clean tube and made ready for digestion with restriction enzymes to check the identity of the plasmid.

b). Large Scale Preparation of Plasmid DNA. The large scale preparation of plasmid DNA was prepared according to a modified alkaline lysis procedure (Birnboim and Doly,

1979). After confirming the identity by restriction analysis of the small scale plasmid DNA preparation, the bacteria was cultured in 200 ml LB at 37° C overnight. The plasmid DNA was purified by using a QIAGEN plasmid purification kit and was loaded into a Centricon-30 concentrator (Amicon, Inc. Beverly, MA), diluted with Milli-Q H₂O and then centrifuged at 3000 rpm for 25 min (2× at room temperature), to remove all the excess salt and concentrate the DNA. The purified plasmid DNA was stored at -20 °C for future use.

3. Isolation of cDNA Inserts

The specific cDNA insert was cut out from the plasmid vector by appropriate restriction enzyme digestion. The mixture of DNA and enzyme solution was fractionated on an agarose gel. After identifying the correct band, the band was cut out and put into a clean dialysis membrane tubing (Spectrum Medical Industries, Inc. Los Angeles, CA.). The DNA fragments of the correct size were then eluted into the dialysis tubing containing 0.5× TAE. The electrodes were reversed briefly (10 sec.) to get any DNA fragments bound to dialysis tubing back into solution. The DNA fragments were collected and underwent further purification as described above (section K-2b).

4. ³²P-Labeling of Hybridization Probes

a). *Labeling Double-Stranded DNA with Random Hexamers.* Human GAPDH cDNA clone and rat SERCA2 cDNA clone to be used as cDNA probes for hybridization were

labeled to high specific activity with (α - ^{32}P) dCTP using the multi-prime DNA labeling method (Feinberg and Vogelstein, 1983). In this method, random oligonucleotides were used to prime DNA synthesis *in vitro* by using denatured linear double-stranded DNA fragments as the template. Over 70% of the precursor triphosphate was routinely incorporated into complementary DNA, and specific activities of over 10^9 dpm/ μg of DNA were obtained.

The multi-prime labeling system was used to label the cDNA probe for Northern blotting analysis. In the 50 μl volume reaction, 100 ng of denatured DNA fragments was used as the template. The reaction mixture also contained buffer, primer, equal molar quantities of unlabeled dGTP, dATP, dTTP, 50 μCi (α - ^{32}P) dCTP and 5 U Klenow DNA polymerase enzyme. The reaction mixture was gently mixed and incubated at 37° C for 30 min. The reaction was stopped by adding 2 μl of 0.5 M EDTA and chilled on ice. Before the probe was added into the hybridization tube, radio-labeled DNA fragments were denatured by heating at 100° C for 3 min with subsequent chilling on ice.

b).5'-end labeling of DNA oligonucleotides. 40-base oligonucleotide probes specific for the 3' untranslated regions of the rat MHC- α and MHC- β genes were used to quantify MHC mRNA levels (Pope et al., 1980). An oligonucleotide probe specific for rat 18S rRNA was used for verifying equal loading conditions of Northern blots in the BDM experiment and actinomycin D experiment. The oligonucleotide probes were end-labeled with (γ - ^{32}P) ATP using T4 polynucleotide kinase. In the 30 μl volume reaction, 240 pmole of oligonucleotide was mixed with kinase buffer (10 \times buffer: 50 mM Tris-HCl,

pH 8.0, 10 mM MgCl₂, and 5 mM dithiothreitol (DTT)), 20 µCi (γ- ³²P) ATP and 10 U T₄ Kinase. The reaction mixture was gently mixed and incubated at 37° C for 60 min. The reaction was stopped by adding 2 µl of 0.5 M EDTA and chilled on ice.

5. Quantification of ³²P Incorporation

To test the amount of ³²P incorporation, two 1 µl aliquots of the labeled reaction mixture were added to 2 pieces of Whatman filter paper separately. One of them was washed in 0.5 M Na₂HPO₄ solution twice (5 min/each) and then washed in Milli-Q H₂O for 5 min. The washed and unwashed filter papers were counted in a WALLAC BETA counter. The percent of ³²P incorporation was calculated by: cpm of washed filter/ cpm of unwashed filter. The incorporation rates were generally greater than 70 %. The probe was added to the hybridization tube to the final concentration of 10⁶ incorporated cpm/ml.

L. POLYMERASE CHAIN REACTION

To analyze the effect of PMA and verapamil on the rate of SERCA2 gene expression, a fragment of the SERCA2 promoter region was generated by using two oligonucleotide primers (Primer 1: 5'- GGAAGCTTCC TCCCCTTGGT TGCTG-3', Primer 2: 5'- GGCCATGGAC AGCGGCGGAG GAAACTG-3' corresponding to the region -556/+60 bp relative to the transcriptional start sites of the rat SERCA2 gene (Figure 3). The

primers included HindIII and NcoI restriction sites, respectively, to allow for orientation-specific ligation into a promoterless luciferase expression plasmid (pluc, kindly provided by Dr. Kaie Ojamaa, Cornell University Medical College). PCR amplification was performed in a 50 μ l volume containing 1 μ g genomic DNA, Taq DNA polymerase 2.5 U, amplification primer pair (100 pmol for each) and PCR buffer (pH 8.5, MgCl₂, 22.5 mM). Samples were overlaid with 50 μ l mineral oil to prevent evaporation and were incubated at 94° C for 3 min to allow the first denaturation, followed by the following cycling parameters for amplification: 94° C, 1 min, 55° C, 30 sec, and 72° C, 2 min. A total 35 amplification cycles were performed. After 10 min of final extension at 72 °C, the PCR products were analyzed by agarose gel electrophoresis, which demonstrated a single fragment of 616 bp.

M. DNA SUBCLONING

1. Restriction Enzyme Digestion

To subclone the rat SERCA2 gene promoter DNA into reporter plasmid pluc, the 616 bp PCR product and the pluc plasmid were sequentially cut with HindIII and NcoI. The cut PCR product was further purified using a Centricon-30 concentrator as described above (section K-2b). The cut plasmid mixture was loaded on an agarose gel and the

5734 bp fragment of the plasmid DNA was collected as promoter vector (detailed procedure see section K-3).

2. Ligation and Transformation

Ends-compatible insert and vector were ligated together by T4 DNA ligase under the following conditions. In a total volume of 30 μ l, 150 ng vector was mixed with insert in a 1:3 molar ratio in the presence of 50 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 1 mM ATP, 1 mM DTT, 5% (w/v) polyethylene glycol-8000, and 1 U T4 DNA ligase. The ligation reaction mixture was incubated at 16° C overnight (Figure 4) and yielded pSERCA-luc.

The pSERCA-luc plasmids were then transformed into *E.Coli* bacteria as described above (section K-1). Then a small scale plasmid preparation was made to identify the plasmid. Finally, a large scale plasmid preparation was made and purified for sequencing and transfection assay.

Illustration for SERCA2 Promoter Transfection Assay

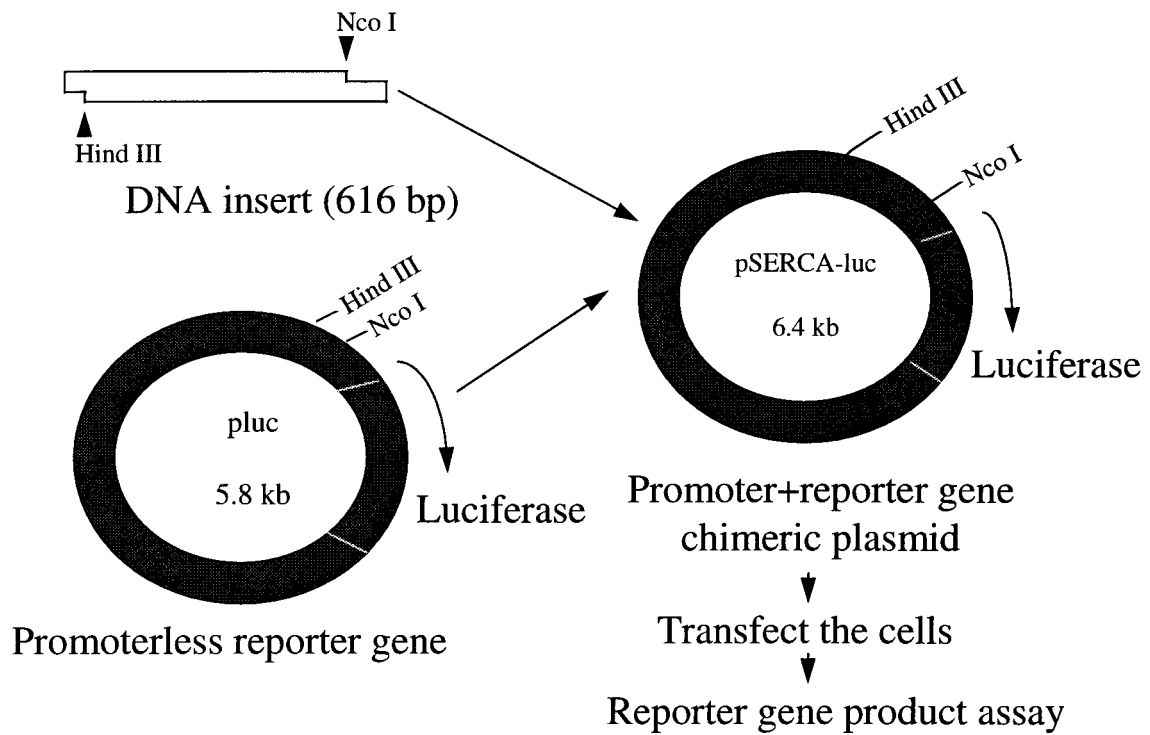


Figure 4: Illustration of the SERCA2 promoter transfection assay protocol.

N. DNA SEQUENCING

The inserted promoter was sequenced using a sequenase version 2.0 DNA sequencing kit based on the chain-termination DNA sequencing method (Sanger et al., 1977). The sequencing strategy used in this study was to sequence this 616 bp PCR product from both DNA strands using two oligonucleotide primers encoding the pluc plasmid sequences from 5' up-stream and 3' down-stream of the promoter insert, respectively. In this case, PA-3 (5' TGGCAGATTA TGATCAG 3') and LU-1 (5' ATGCAGTTGC TCTCCAGCGG TT 3'), kindly provided by Dr. Kaie Ojamaa (Cornell University Medical College), were used to sequence the positive and negative strand of this PCR product respectively. A 17 bp primer (5'GCCGCGACCG CGTAAGG 3' obtained from Dr. Bassam Wakim, Loyola University Medical Center) corresponding to the rat SERCA2 promoter region -310/-293 was also made to sequence the middle part of this DNA fragment. The sequencing procedure was outlined as following. Supercoiled plasmid DNA was denatured by a 0.2 M NaOH, 0.2 M EDTA solution for 30 min at 37° C and neutralized by adding 0.3 M Na Acetate solution, pH = 5.2. The denatured DNA was pelleted and air-dried and resuspended in a given volume of Milli-Q water. To anneal denatured plasmid DNA with sequencing primer, 2 µl of sequencing buffer was added to the 7 µl of DNA and 1 µl of primer (10 ng/µl) and incubated at 37 °C for 30 min. To label the primers, 1 µl of 0.1 M DTT, 1 µl of ³⁵S-αdATP, 2 µl of dilute labeling solution and 2 µl of dilute sequenase version 2.0 T₄ DNA polymerase were added to the

annealed reaction mixture and incubated at room temperature for 10 min. After labeling, 3.5 μ l of the above mixture was added to the side of each termination tube which contains d/ddNTP. The mixture was incubated at 37° C for 10 min. The reaction was stopped by adding 4 μ l of stop solution and cooled immediately on ice. The samples were denatured at 80° C for 5 min immediately before loading. Half of the reaction volume was then loaded on a 6 % polyacrylamide sequencing gel (15 ml 40 % acrylamide stock solution, 20 ml 5 \times TBE, 25 ml dH₂O, 5 g urea, 1 ml 10% APS, 35 μ l TEMED). The gel was run at 55 W for 2 h with 1 \times TBE buffer using a Jordan Scientific Co. sequencing apparatus. The second half of the reaction was loaded on the gel for another 2 h electrophoresis. At the end of electrophoresis, the gel was fixed in 10 % acetic acid and 10 % methanol for 15 min. After fixation, the gel was adhered to a 3 MM Whatman paper, covered with Saran wrap, dried at 80° C for 30 min, then exposed to an X-ray film.

O. TRANSIENT TRANSFECTION ASSAY.

1. Transfection

For DNA transfection, two plasmid constructs were used. pRSVlacZ, which contains the *E Coli* β -galactosidase gene under control of a rous sarcoma virus (RSV) enhancer was used as an internal standard for normalization of transfection efficiency. pSERCA-luc, as the promoter-reporter gene construct plasmid, contains the promoter element of

the SERCA2 gene and a promoter-less reporter gene encoding firefly luciferase. Neonatal rat myocytes were plated onto 6 well dishes (1.2×10^6) and maintained overnight in PC-1 medium. The DNA solution was prepared by adding pSERCA-luc (5 $\mu\text{g}/\text{well}$) and pRSV β gal plasmid (0.5 $\mu\text{g}/\text{well}$) into Optimem I solution to a final volume of 100 $\mu\text{l}/\text{well}$. Lipofectamine solution was prepared by adding 15 $\mu\text{g}/\text{well}$ of Lipofectamine into Optimem I solution in a final volume of 100 μl . Then the DNA solution was mixed with lipofectamine solution and incubated at room temperature for 15 min. The liposome-DNA mixture was diluted with 2:1 medium containing 12 $\mu\text{g}/\text{ml}$ gentamicin and then the liposome-DNA mixture was added to a triplicate set of plates. The cells were cultured in antibiotic-free medium for 6 h and then maintained in normal growth medium with or without PMA or verapamil for 48 h.

2. Harvesting

The transfected cells were then washed with ice-cold HBSS, and 500 μl of lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 10 % glycerol, and 1 % Triton X-100) was added. Cells were scraped off the dishes, and the lysate was centrifuged at 12,000 rpm for 10 min in a cold room to remove insoluble debris. The supernatant was collected and placed on ice for the luciferase and β -galactosidase assay.

3. Luciferase Assay

The luciferase assay was performed as described (Ojamaa and Klein, 1991). 100 μ l of sample was added to 500 μ l 2 \times luciferase assay buffer (50 mM Tris-acetate pH 7.8, 10 mM magnesium acetate, 2 mM ATP, 25 mM DTT, 1 mM EGTA). 300 μ l of water and 100 μ l of luciferin (2 mM) was automatically injected into the sample immediately before counting. Luminescence was measured in a luminometer.

4. β -galactosidase (β -gal) Assay

β -gal activity in the cell extracts was assayed according to the method of Miller, with o-nitrophenyl- β -D-galactopyranoside (ONPG) as the substrate (Miller, 1992). 200 μ l of sample was mixed with β -gal assay buffer (2.5 \times β -gal assay buffer: 48 mM Na₂HPO₄, 32 mM NaH₂PO₄, 8 mM KCl, 0.8 mM MgCl₂ and 14.28 μ l/10 ml of β -mercaptoethanol) and 200 μ l of ONPG to make a final volume of 1 ml and incubated in 37° C water bath for 3 hours. The reaction was stopped with 200 μ l of 2M Na₂CO₃ and vortexed. The calorimetric reaction was detected at 420 nm in a spectrophotometer using water as the blank.

5. Expression of Data

Luciferase activity was presented as background-subtracted units divided by the control β -gal activity to normalize for the differences in transfection efficiency.

P. ACTINOMYCIN D (act-D) mRNA STABILITY ASSAY.

The relative stability of SERCA2 mRNA in control and verapamil or PMA treated cells was assessed following treatment of myocytes with act-D. Neonatal myocytes were maintained in growth medium in the absence or presence of PMA (200 nM) or verapamil (10 μ M) for 24 h. Act-D (5 μ g/ml) was then added, and the cells maintained for an additional 0, 12 or 24 h at which time total RNA was isolated by the method of Chomzynski and Sacchi (1987). RNA was quantified, size-fractionated and analyzed by Northern blotting with probes specific for SERCA2 and 18 S rRNA as described above. Band intensity was quantified by laser densitometry.

Q. DNA AND PROTEIN ASSAY.

Total cellular protein and DNA content of the samples (from LV tissue and cultured neonatal rat ventricular myocytes) were measured by the Lowry (Lowry et al., 1951) and Bisbenzimid assays (Cesarone et al., 1979), respectively. The cells on 35 mm dishes were washed twice in HBSS, and 0.2 N perchloric acid (1 ml) was added to the culture well. The precipitated macromolecules were then quantitatively scraped from the dishes, and collected by centrifugation (10,000 g; 10 min). The precipitate was washed with 0.2

N perchloric acid once and redissolved by incubation (37° C, 20 min) in 1 ml of 0.3 N KOH. Aliquots were then used for analysis of total protein by the Lowry method using crystalline human serum albumin as a standard. For DNA assay, 33258 Hoechst dye and salmon sperm DNA standard were used in Bisbenzimid assay. Data are the means of duplicate or triplicate wells from each treatment group of each cell isolation, and were expressed as µg/dish. For tissue samples, 5 % of tissue homogenate was made in 0.9 % saline solution and a 50 µl aliquot of the sample was used for the Lowry protein assay.

R. QUANTITATIVE ANALYSIS OF TOTAL MHC AND MHC ISOENZYMES.

1. Quantification of Total MHC Protein by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

For quantitative analysis of total MHC (MHC- α and MHC- β) protein content, cells in 35 mm dishes were washed twice in HBSS and lysed in 500 µl of Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, containing 0.01% bromophenol blue, 8% SDS, 5% 2-mercaptoethanol, 10% glycerol). The protein samples were denatured by heating to 100° C for 20 min. Equal volumes (80 µl) were loaded onto 2.7 mm × 180 mm SDS-PAGE gradient (7.5-17%) gel along with additional lanes containing known amounts (1-10 µg) of purified cardiac MHC. The gel was run in SDS gel running buffer (25 mM Tris, 192 mM glycine, pH 8.3, containing 0.1% SDS) at 12.5 mA per gel, 12° C overnight until the

bromophenol blue dye marker migrate to the bottom of the gel and then stained in 0.1 % Coomassie brilliant blue (CBB, 0.1 g CBB in 100 ml methanol: H₂O: acetic acid, 5:5:2). Band intensity was quantified by laser densitometry and compared to the band intensity of purified MHC standards. Results were the means of duplicate wells from each treatment group for each cell isolation, and expressed as µg MHC/dish.

2. Quantification of MHC Isoenzymes by SDS-PAGE and Silver Staining

To quantify the relative proportions of MHC- α and MHC- β isozymes in LV tissue and cells, equal volumes (15 µl) of protein samples prepared in Laemmli sample buffer were loaded onto a 5 % acrylamide SDS-PAGE gel along with a known amount of protein rainbow standard. The gel was run at 12° C, 35 mA/per gel for 3 h until the MHC band in the rainbow standard runs to the 2/3 of the length of the gel. The gel was carefully taken off and soaked in 50 % methanol overnight. The next day, the gel was stained using modified silver staining technique (Guevara et al., 1982). Band intensity was quantified by laser densitometry.

S. WESTERN BLOT ANALYSIS.

For quantitative analysis of SR Ca ATPase protein content, samples were prepared in Laemmli sample buffer as described as above. Equal amounts of protein (20 µg

protein/lane) were loaded onto and electrophoresed through a 7.5% SDS-polyacrylamide gel at 100 Volts in SDS gel running buffer for about 1.5 h until the bromophenol blue dye marker migrated to the bottom of the gel. Proteins were electroblotted to nitrocellulose sheets in 1 × SDS-PAGE running buffer with 20 % of methanol at 80 V for 1h (Towbin et al., 1979). The blot was prepared for antibody analysis by soaking in blocking solution (5% non fat milk solids (NFMS) and 1× TBST [10× TBST: 0.1 M Tris-HCl, pH 8.0, 0.75 % Tween 20 and 1.5 M NaCl]) overnight at 4° C. Rabbit anti-rat SERCA2 antiserum was diluted 1:1000 in TBST with 5 % NFMS. The blot was incubated with primary antiserum solution for 1 h at room temperature, rinsed with TBST and washed in TBST for 5 min 3 times, then incubated in TBST containing 5 % NFMS for 15 min. The secondary antibody, anti-rabbit IgG conjugated with horseradish peroxidase (diluted in blocking solution 1:5000) was added to the blot and incubated at room temperature for 1 h. The blot was washed as above, then placed on a glass plate. After removing all the air bubbles, equal amounts of detecting solutions from the Chemoluminescence kit were added onto the blot and immediately covered with saran wrap for 1 min. The film was developed after placement on top of the covered blot for 1-5 min. The band intensity was quantified by laser densitometry.

T. DATA ANALYSIS.

All results were expressed as means \pm SEM. Data were analyzed using the PROPHET computer system software (Division of Research Resources, NIH). The data were tested for normality by Wilk-Shapiro test followed by Levene's test to examine the equality of variances. If the data passed both tests, one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test were used for multiple comparisons and two-way ANOVA was used to test the factors "PMA" and "contraction" and their interaction on measurements of growth and gene expression. If the data were not normally distributed, or if the variances were not homogeneous, then the Kruskal-Wallis test followed by Dunn's test was used for comparisons among sham, 8 wk banded and 16 wk banded groups in the whole animal study and Friedman repeated measures analysis of variance on ranks followed Student-Newman-Keuls test was used to analyzed the effects of verapamil and PMA on the SERCA2 promoter activities in the transient transfection study. Nonlinear regression analysis was used to estimate the half life of SERCA2 mRNA in the message stability assay. The paired and unpaired t-tests were also used for comparisons of parametric data between two groups. In each case, significance was accepted at $p < 0.05$.

CHAPTER IV

RESULTS

A. DO CHANGES IN SERCA2 GENE EXPRESSION AFFECT CARDIAC PERFORMANCE DURING LVH PROGRESSION?

1. Abdominal AoC Produced Hypertension and LVH.

A total of 45 male Sprague-Dawley rats (14 sham-operated sham and 31 banded rats) were used in this study. Surgical procedures were conducted on 3 separate occasions. Perioperative mortality (defined as mortality within 72 h of surgery) was 0% in the sham-operated group, but ranged from 0 to 30% in the banded group. Thereafter, 100% of the sham-operated animals and approximately 80% of the banded animals survived until evaluation at either 8 or 16 weeks. Both sham and banded animals were evaluated at each of these time points, but since none of the morphometric, hemodynamic or biochemical measurements differed significantly between 8 wk and 16 wk sham rats, the data from all sham animals were pooled for the sake of clarity.

As seen in Table 5, coarctation of the abdominal aorta produced significant increases in systolic arterial pressure (SAP), diastolic arterial pressure (DAP) and mean

arterial pressure (MAP) measured 8 wk after banding, as compared to sham-operated controls. Thereafter, blood pressure measurements did not change significantly.

Sustained hypertension was associated with the development of LVH. The LV/body weight ratio was 38% greater in 8 wk banded rats as compared to sham-operated animals (2.15 ± 0.15 and 2.96 ± 0.17 mg/g for sham and 8 wk banded rats, respectively; $P < 0.01$). The LV/body weight ratio was also significantly ($P < 0.01$) increased (by 45%) in 16 wk banded animals (3.13 ± 0.14 mg/g) as compared to sham animals. However, LV/body wt ratio was not statistically different between 8 and 16 wk banded animals.

2. Abdominal AoC Produced Alterations In Cardiac Performance.

I next evaluated whether sustained hypertension was associated with the development of systolic and/or diastolic dysfunction over time. High-fidelity recordings of LV pressure were obtained using a manometer-tipped, 3F catheter advanced from the right carotid artery to the LV chamber, and the digitized data analyzed with respect to indices of systolic and diastolic performance. As seen in Table 5, $+dP/dt_{\max}$ and contractility index (CI) were not significantly altered in either 8 wk or 16 wk banded animals, indicating that isovolumic systolic function was relatively normal despite increased afterload and LVH. In contrast, the banded rats demonstrated abnormalities in diastolic function, especially at 16 weeks. The $-dP/dt_{\max}$ was decreased in both 8 wk and 16 wk banded rats, but these differences did not attain statistical significance. Since $-dP/dt$ is afterload-dependent (Lorrell, 1991), individual values from the 3 groups were normalized

by dividing by the MAP, as previously described (Burgess et al., 1995). As seen in Table 5, normalized $-dP/dt_{\max}$ was significantly ($P<0.01$) reduced in both 8 wk and 16 wk banded animals, but there was no significant difference noted between 8 and 16 wk banded rats. The relaxation time constant τ was also prolonged in both 8 and 16 wk animals however, statistical significance was only found in 16 wk banded rats ($P<0.01$) as compared to sham-operated controls.

In contrast, LVEDP was relatively normal in 8 wk banded rats, but was significantly ($P<0.01$) elevated in 16 wk banded animals. The elevated LVEDP indicated that cardiac performance in 16 wk banded animals had deteriorated without a further increase in LV mass, worsening hypertension, or evidence of isovolumic systolic dysfunction. Thus, these 16 wk animals were considered to be in a “transitional” phase between stable or compensated LVH and heart failure.

The observed increases in τ and LVEDP in pressure-overloaded animals were not the result of acute changes in afterload, but were dependent upon changes in myocardial structure or function that developed over time. As seen in Figure 5, partial occlusion of the abdominal aorta of a normal rat at the identical site of aortic banding caused an acute increase in systolic arterial pressure, but had no effect on either τ (Panel A) or LVEDP (Panel B).

TABLE 5

Hemodynamic Measurements In Sham and Banded Rats

	Sham	8 wk-Banded	16 wk Banded
SAP (mmHg)	130±6 (n=14)	176±15* (n=9)	195±6* (n=22)
DAP (mm Hg)	88±5 (n=14)	111±9* (n=9)	120±3* (n=22)
MAP (mm Hg)	102±5 (n=14)	133±11* (n=9)	145±4* (n=22)
+ dP/dt _{max} (mm Hg/sec)	8057±411 (n=14)	9078±1240 (n=9)	7921±363 (n=22)
Contractility Index	115±3 (n=10)	113±16 (n=8)	101±8 (n=16)
- dP/dt _{max} (mm Hg/sec)	7411±346 (n=14)	6944±818 (n=9)	6386±322 (n=22)
(-dP/dt _{max})/ MAP (sec ⁻¹)	74±3 (n=14)	54±6* (n=9)	46±2* (n=22)
τ (msec)	14.5±0.8 (n=10)	25.7±5.8 (n=8)	25.1±2.0* (n=14)
LVEDP (mm Hg)	3.6±1.0 (n=14)	7.9±3.1 (n=9)	15.2±2.1* (n=21)

* P < 0.05 vs. Sham.

SAP = Systolic arterial pressure.

DAP = Diastolic arterial pressure.

MAP = Mean arterial pressure.

LVEDP = Left ventricular end diastolic pressure.

τ = The time constant for isovolumic relaxation.

Contractility Index = (+dP/dt_{max})/LVP at peak dP/dt.

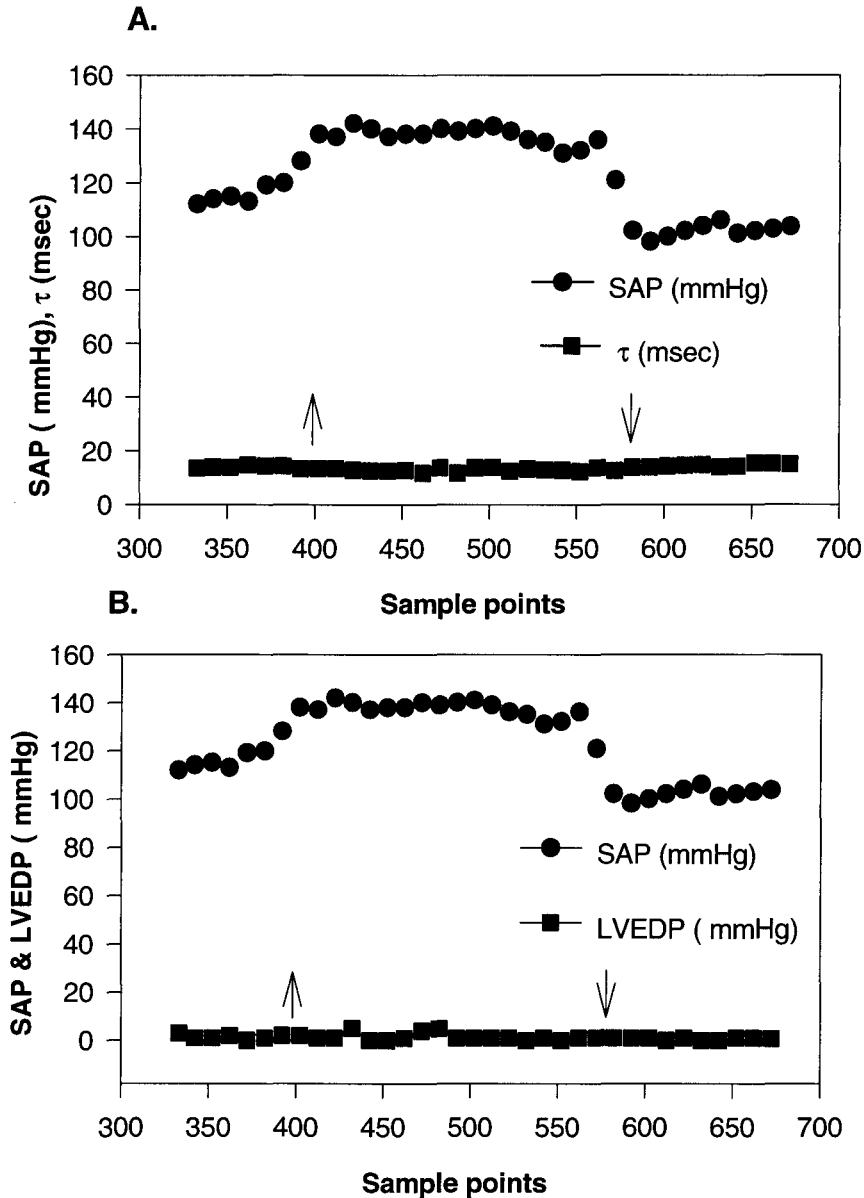


Figure 5: SAP, LVEDP and τ before and after acute aortic banding. A normal rat was anesthetized with ketamine-xylazine. Abdominal surgery and carotid artery cannulation were performed as described in the Methods section. A snare was placed around the identical site as used for aortic banding. \uparrow and \downarrow represent the time at which I tightened and loosened the snare, respectively.

3. Changes In Tissue Composition Coincide With The Development Of Progressive LV Dysfunction.

A growing body of evidence suggests that down-regulation of SERCA2 gene expression is the most important contributor to the relaxation abnormality during pressure overload hypertrophy and CHF. However, there is lack of evidence which directly associates diastolic function with changes in SR Ca^{2+} ATPase mRNA and protein levels during the development of LVH. Therefore, I examined whether alterations in SERCA2 expression coincided with the appearance of diastolic dysfunction during pressure overload. As seen in Figure 6A, SERCA2 protein levels in 8 wk banded rats were not significantly different from sham-operated animals, when equal amounts of total protein from LV tissue homogenates were compared by Western blotting. In contrast, I found a significant reduction ($P<0.05$) in SERCA2 protein levels in LV tissue homogenates of 16 wk banded rats as compared to 8 wk banded animals. Because the increase in ECM collagens (details see next paragraph) may have contributed to dilution of SERCA2 protein within the complex mixture of total tissue proteins, homogenates were prepared from freshly isolated myocytes of sham and 16 wk banded animals, and also compared by Western blotting. As seen in Figure 6B, SERCA2 protein levels from 16 wk banded rats were also significantly ($P<0.05$) reduced to 66% of control levels in these homogenates.

The decrease in SERCA2 protein levels in 16 wk banded animals was associated with an even greater reduction in SERCA2 mRNA levels, as analyzed by Northern blotting. As seen in Figure 7, SERCA2/GAPDH mRNA ratios in 16 wk banded animals were only

24±9% of those observed in sham-operated control animals ($P<0.01$). Interestingly, SERCA2/GAPDH mRNA levels were also significantly ($P<0.01$) reduced to 43±10% of control rats 8 wk after banding, despite the fact that SERCA2 protein levels were unchanged at this time point.

Above data indicates that the down-regulation of SERCA2 gene was not the primary cause for the relaxation abnormality in 8 wk hypertensive animals. However, down-regulation of SERCA2 gene expression predicted the onset of overt deterioration of cardiac performance during the development of pressure overload LVH as evidenced by decreases in SERCA2 mRNA levels prior to markedly elevated LVEDP and a significantly prolonged τ . The reduction in SERCA2 mRNA and protein levels that coincided with the significant increases in LVEDP and τ after 16 wk aortic banding indicates that down-regulation of SERCA2 gene expression indeed played an important role in deterioration of cardiac function during the progression of pressure overload LVH.

Additional factors must contribute to the relaxation abnormalities that occurred in the compensated LVH. Since increase in ECM collagen content has been suggested to responsible for the diastolic dysfunction in chronic hypertensive rats (Burgess et al., 1995 in press), I next examined whether alteration of hydroxyproline contents was temporally associated with the development of impaired cardiac relaxation in these hypertensive animals. As seen in Figure 8, I noted 72 % and 49% increase in the amount of protein-bound hydroxyproline in LV tissues of 8 and 16 wk hypertensive rats, respectively. and It indicated significant ($P<0.05$) increases in the amount of myocardial collagens 8 and 16

wk after aortic banding. However, there was no significant difference in hydroxyproline content between 8 and 16 wk banded animals (Figure 8). These results suggests that increases in collagen content may account in part for the impaired cardiac relaxation that occurred after 8 wk of hypertension. However, it may not be related to the deterioration of cardiac performance during the progression of LVH.

Since a switch in MHC isoenzyme predominance from MHC- α to MHC- β is characteristic of pressure overload LVH in the rat (Mahdavi and Nadal-Ginard, 1989), the percentage of MHC- β isoenzyme in LV tissue homogenates was quantified by SDS-PAGE, silver staining, and laser densitometry at 8 and 16 wk after AoC. As seen in Figure 9, $30\pm3\%$ of the MHC expressed in ventricular muscle of the sham-operated animals was MHC- β . The relative concentration of MHC- β was not significantly increased in 8 wk banded rats, but accounted for $59\pm3\%$ of the total MHC 16 wk after aortic banding ($P<0.01$ vs. sham and 8 wk banded animals, respectively). These results indicates that MHC isoenzyme switch was unlikely responsible for the impaired cardiac relaxation occurred in 8 wk hypertension. However, it may in part responsible for the deterioration of cardiac performance during progressive LVH.

In summary, although my original hypothesis was incorrect, my data are indeed consistent with an important contribution of SERCA2 down-regulation to the progressive deterioration in LV performance over time. Therefore, further studies are required to determine the intracellular mechanisms responsible for SERCA2 down-regulation during LVH progression, and to elucidate the specific molecular events responsible for progressive diastolic dysfunction in the pressure overloaded heart.

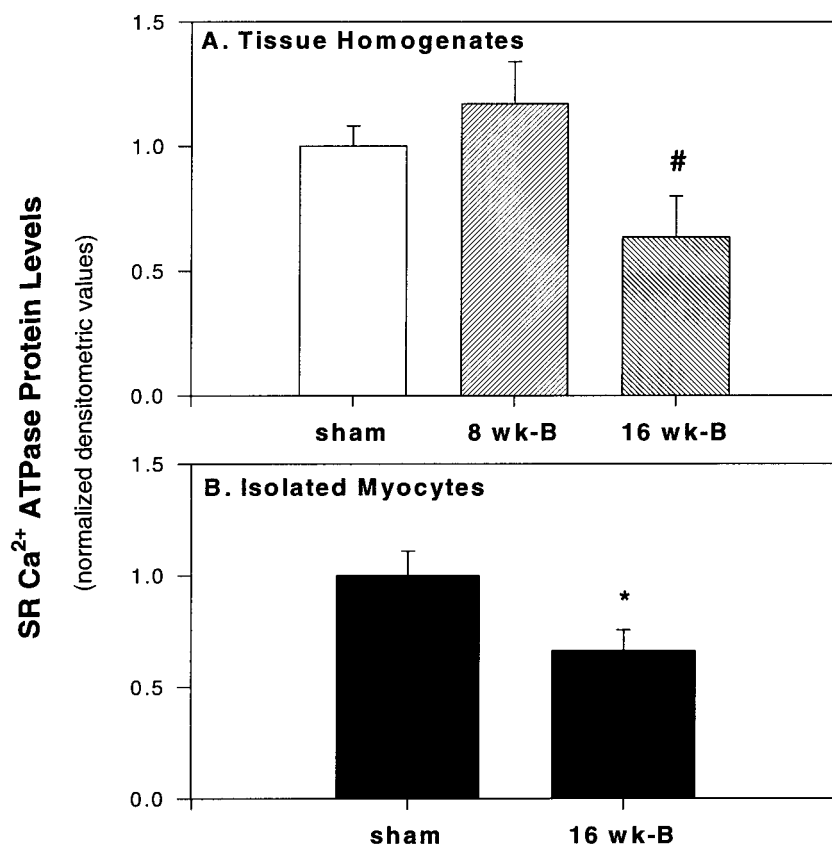


Figure 6: SR Ca ATPase protein levels in sham and banded rats. Tissue homogenates (panel A) or isolated myocyte homogenates (panel B) were diluted 4 fold with 8 % Laemmli buffer. Equal amounts of protein (20 μ g/lane) were separated on 7.5 % SDS-polyacrylamide gel. SR Ca ATPase protein levels were quantified by Western blotting using anti-rat SERCA2 antiserum. Data were normalized with the mean value of bands in sham groups in each gel. Data are the mean \pm SEM * $P < 0.05$ vs sham, # $P < 0.05$ vs 8 wk-B. B= banded by 1-way ANOVA followed by Student-Newman-Keuls test (Panel A), and unpaired t-test (Panel B).

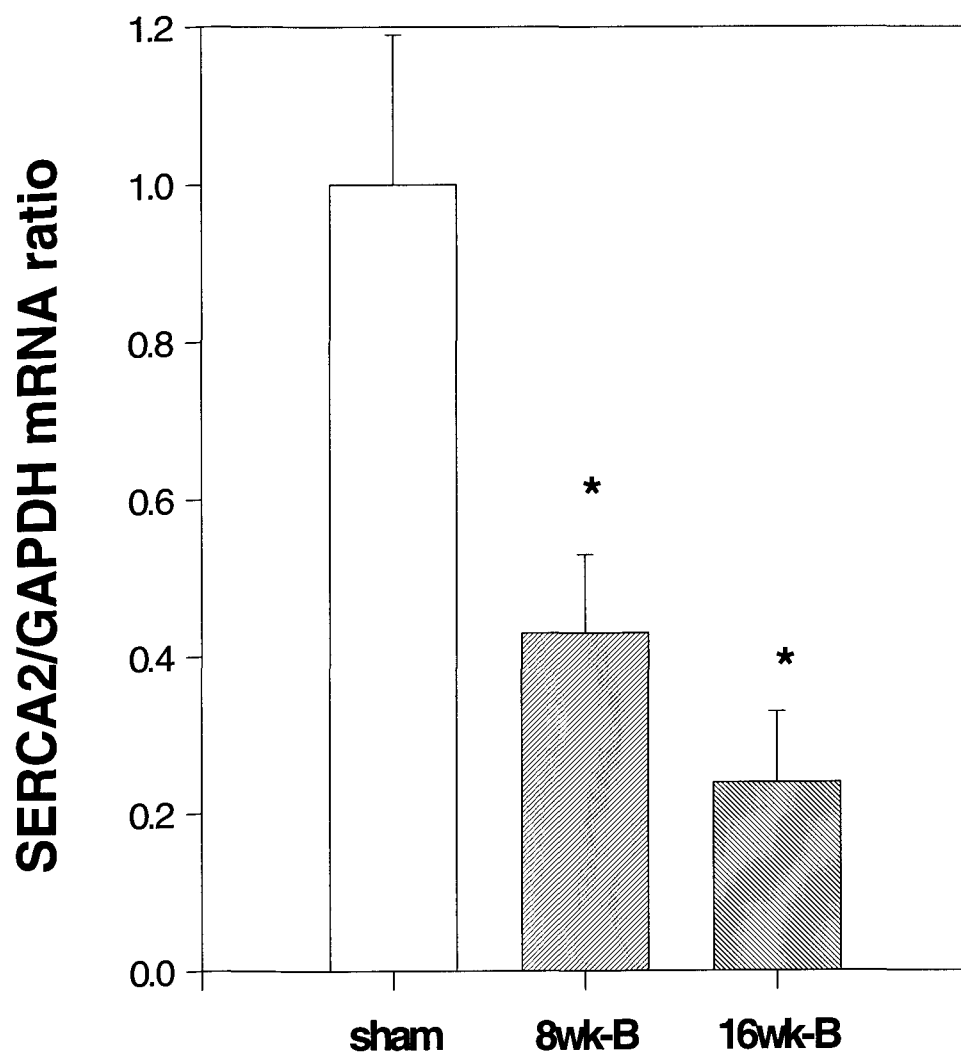


Figure 7= Quantitative analysis of SERCA2 mRNA levels in sham and banded rats. RNAs were isolated from sham, 8 and 16 wk banded rats. SERCA2 mRNA levels are normalized to GAPDH mRNA and plotted as percent of the mean values in sham groups. * $P < 0.05$ vs sham. B= Banded.

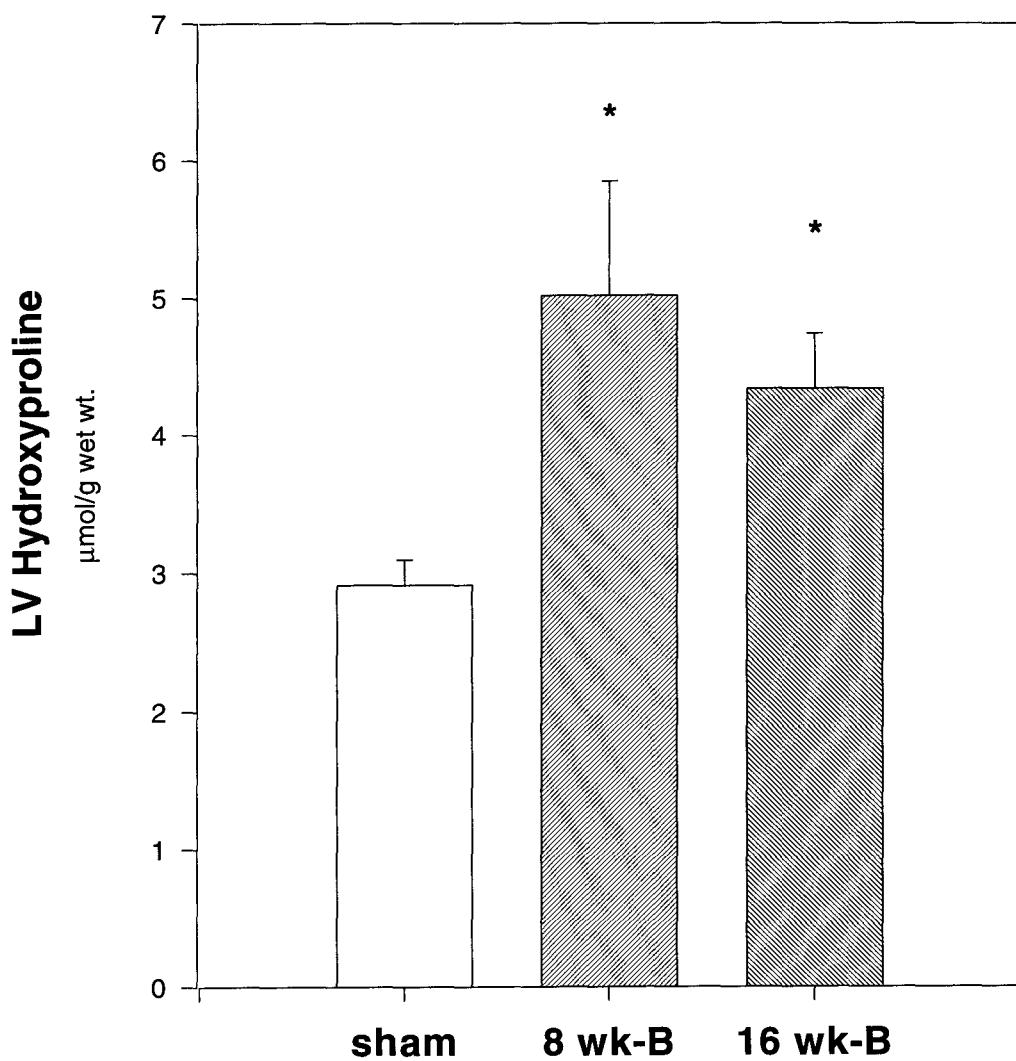


Figure 8: Left ventricular (LV) hydroxyproline contents in sham and banded rats. Data are the mean \pm SEM. N= 5, 4 and 15 animals in sham, 8 wk-B and 16 wk-B groups, respectively. Data were compared by Kruskal-Wallis test followed by Dunn's test. * P < 0.05 vs sham. B = banded.

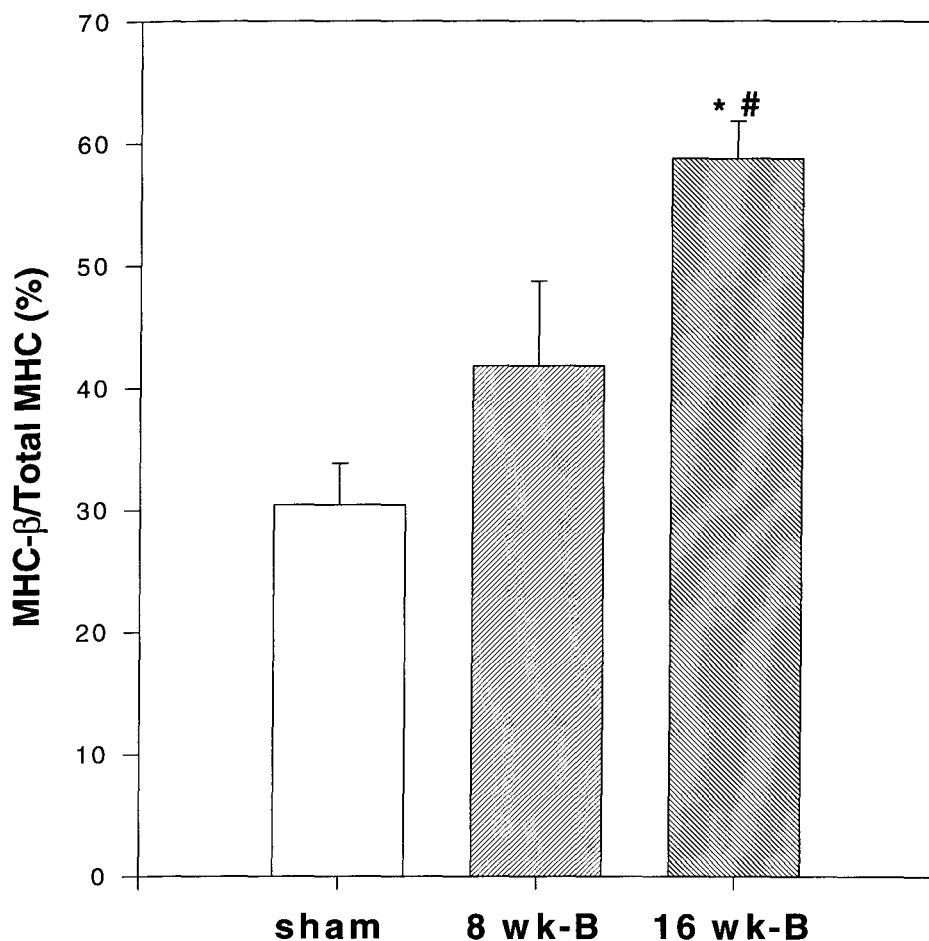


Figure 9: MHC- β isoenzyme in sham and banded rats. Tissue homogenate was diluted 4-fold with 8 % Laemmli sample buffer. Equal volumes (15 μ l) of diluted tissue homogenates were separated on 5 % SDS-polyacrylamide gels and the gels were stained by silver-staining technique. MHC- α and MHC- β band intensities were quantified by laser densitometry. Data are expressed as MHC- β /total MHC (MHC- α +MHC- β). Data are the mean \pm SEM. N= 13, 4 and 10 in sham, 8 wk-B and 16 wk-B groups respectively. Data are compared by one-way ANOVA followed by the Student-Newman-Keuls test. * P< 0.05 vs sham, # P< 0.05 vs 8 wk-B. B= banded.

B. REGULATION OF SERCA2 GENE EXPRESSION IN CULTURED NEONATAL RAT VENTRICULAR MYOCYTES

1. Neonatal Rat Ventricular Myocyte Cell Isolation and Culture.

The collagenase digestion procedure was used to isolate neonatal rat ventricular myocytes and routinely yielded $3-4 \times 10^6$ cells per neonatal rat heart. Spontaneous contractile activity was visible within 24 h of plating. Within 48 h, islands of cells maintained in growth medium were observed to contract synchronously at a rate of 80-100 beats/min. More prolonged culture lead to further cell spreading so that entire plates or wells were seen to beat synchronously within 72-96 h after plating. The number of nonmuscle cells appeared to increase only minimally during more prolonged incubation of these dense cultures maintained in nutrient-rich, serum-free medium.

2. Up-regulation of SERCA2 Gene Expression by Inhibition of Contractile Activity

a). Up-regulation of SERCA2 mRNA and protein levels by verapamil. To determine whether the inhibition of contractile activity regulates SERCA2 mRNA levels in primary cultures of neonatal ventricular myocytes, spontaneous contractile activity was completely inhibited by adding the L-type calcium channel blocker verapamil (10 μ M, 48 h) to the serum-free culture medium. Verapamil arrest produced cardiac myocyte atrophy, as evidenced by a significant decrease in total cellular protein and total protein/DNA ratio (Table 6). Furthermore, verapamil treatment (10 μ M, 48 h-72 h) also decreased MHC content, and the percentage of the total MHC that was MHC- β , as described in a previous paper (Qi et. al, 1994a).

The effects of verapamil treatment (10 μ M, 48 h) on mRNA levels encoding MHC- α , MHC- β and SERCA2 were determined by Northern blotting, as depicted in the

representative Northern blots presented in Figure 10. Quantitative analysis of 6 separate Northern blotting experiments is depicted in Figure 11. In correlation with the changes in MHC isoenzyme protein levels, MHC- α mRNA levels were increased to $200 \pm 24\%$ of control ($n=6$, $P < 0.05$ by paired t-test), whereas MHC- β mRNA levels was reduced to $10 \pm 3\%$ of control ($n=6$, $P < 0.05$ by paired t-test) (Figures 10 and 11). These results are similar to those previously described (Samarel and Engelmann, 1991) and emphasize a switch from MHC- β to MHC- α induced by verapamil which is opposite to that which occurred during the progression of pressure overload-induced LVH *in vivo*. As seen in Figure 10 and 11, SERCA2 mRNA levels were also significantly increased to $166 \pm 10\%$ of control ($n=6$, $P < 0.05$ by paired t-test). The effect of verapamil on SERCA2 protein levels was also examined by Western blotting after 72 h incubation with verapamil ($10 \mu\text{M}$). As shown in Figure 12, the verapamil-induced increase in SERCA2 mRNA levels was accompanied by a significant increase in SR Ca ATPase protein levels ($164 \pm 20\%$ of control, $n=6$ for both groups, $P < 0.05$ by paired t-test; Bassani et. al, 1994).

TABLE 6

Effect of Verapamil on Growth of Neonatal Rat Heart Cells

	C (n=17)	V (n=17)
Total Protein ($\mu\text{g}/\text{mg}$)	315 ± 15	$205 \pm 15^*$
Protein/DNA ($\mu\text{g}/\mu\text{g}$)	52 ± 1	$44 \pm 2^*$
Total MHC ($\mu\text{g}/\text{dish}$)	15 ± 1	$7 \pm 1^*$
% of MHC- β	37 ± 2	$14 \pm 1^*$

Neonatal rat ventricular myocytes were maintained (72 h) in standard culture medium (C) or medium supplemented with 10 μM verapamil (V). Cells were harvested by either perchloric acid precipitation for analysis of total protein and DNA, or SDS-lysis for analysis of MHC content. Data are mean \pm SEM of duplicate 35 mm dishes of each treatment group from 17 different cell isolations. * $P < 0.05$ by paired t-test.

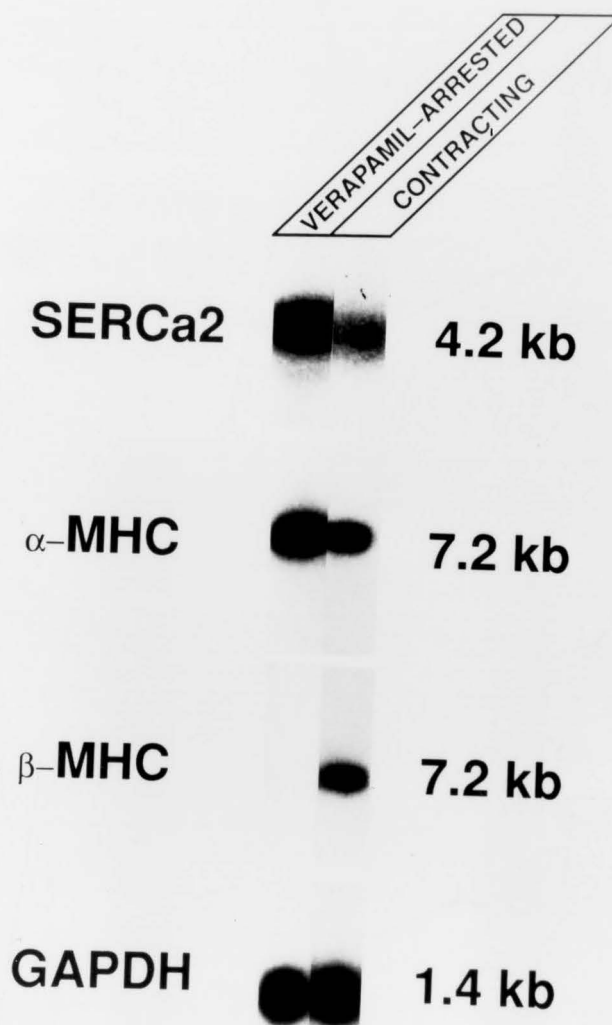


Figure 10: Northern blots for SERCA2, MHC- α , and MHC- β mRNA levels in contracting and verapamil arrested cells. Neonatal rat ventricular myocytes were maintained (48 h) in standard culture medium (Contracting) or medium supplemented with 10 μ M verapamil (Verapamil-arrested). Total RNA was isolated and equal amounts (10 μ g) of RNA were separated on 1% agarose gels and transferred to a nylon membrane. The filter was sequentially hybridized with 32 P-labeled cDNAs and oligonucleotide probes specific for SERCA2, MHC- α , MHC- β and GAPDH genes. Numbers to the right indicate the size of the transcripts in kb. Constitutively expressed GAPDH mRNA levels were used to indicate equality of RNA loading.

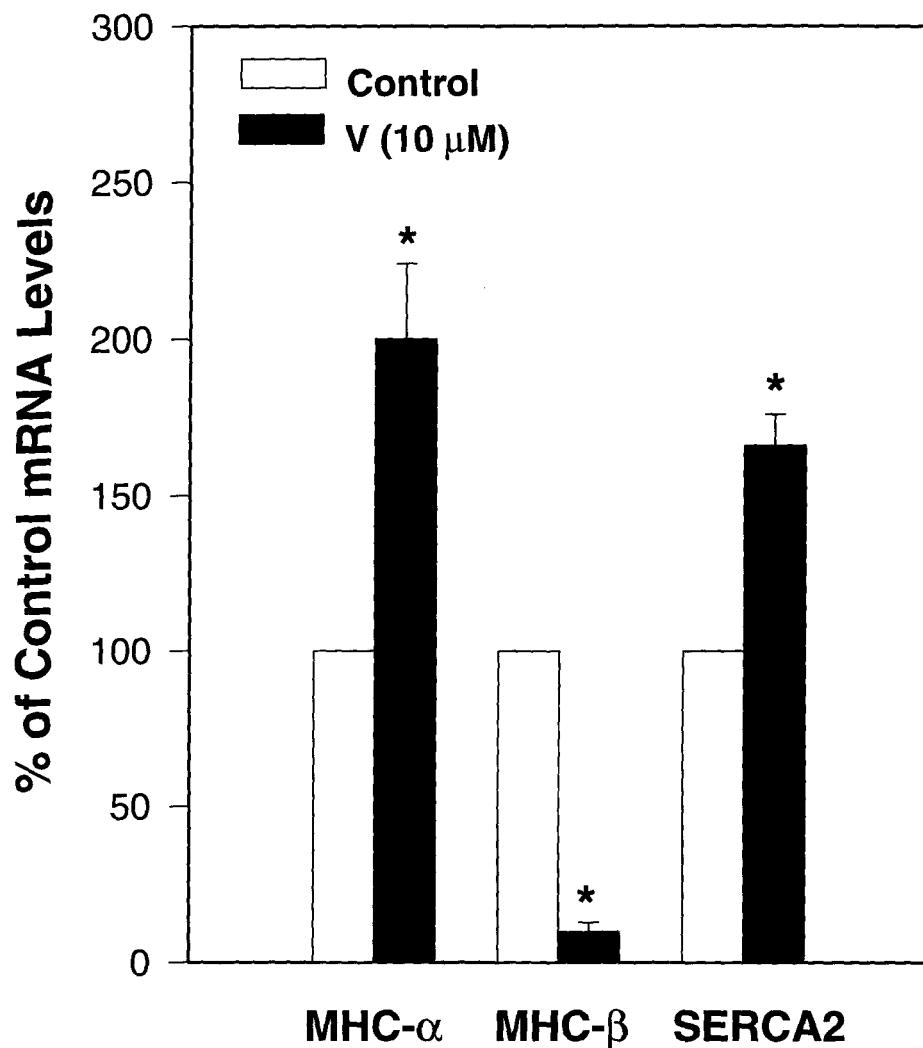


Figure 11: Effects of verapamil arrest on mRNA levels in cultured neonatal rat ventricular myocytes. Neonatal rats ventricular myocytes were maintained (48 h) in standard culture medium (Control) or medium supplemented with 10 μ M verapamil (V). Total RNA was isolated for Northern blotting analysis. The data were obtained from 6 individual Northern blotting experiments, including the autoradiogram depicted in Figure 10. The intensities of MHC- α , MHC- β and SERCA2 mRNA were normalized to the intensity of GAPDH mRNA and plotted as the percentage of the value of contracting (C) cells. * $P < 0.05$ by paired t-test.

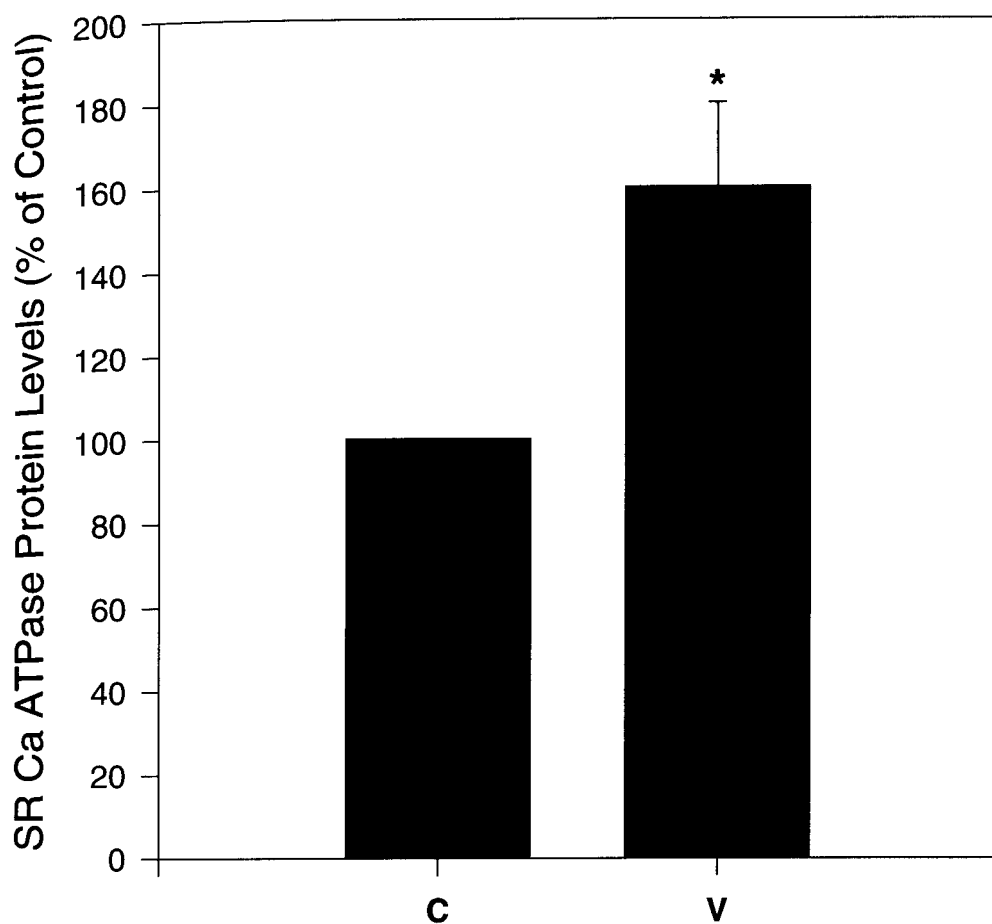


Figure 12: Verapamil induced up-regulation of SERCA2 protein levels. Neonatal rat ventricular myocytes were maintained in serum-free growth medium in the absence (C) or presence of 10 μ M verapamil (V) for 72 h. SERCA2 protein levels were analyzed by Western blotting. The intensities of the bands are normalized as percent of that in C cells. Data are the mean \pm SEM from 6 individual experiments.

b). UP-regulation of SERCA2 mRNA levels by KCl and BDM. An obvious problem to determine the mechanism of verapamil induced up-regulation of SERCA2 mRNA levels is the fact that verapamil not only affects contractile force and amplitude but also eliminates $[Ca^{2+}]_i$ transients in these cultured cells (Barry et al., 1985). To determine whether up-regulation of SERCA2 mRNA levels resulted from the inhibition of contractile activity or alterations in $[Ca^{2+}]_i$, primary cultures of neonatal rat ventricular myocytes were maintained (48 h) under control conditions (C) or in the presence of 10 μ M verapamil (V), 50 mM KCl (KCl) or varying concentration of BDM (5, 7.5 and 10 mM). Like verapamil, high concentration of KCl (50 mM) completely inhibited spontaneous contractile activity. Based upon visual observation of the cultures, BDM at a concentration of 5 mM appeared to reduce both the amplitude and frequency of contractions. The cells were arrested with occasional spontaneously contractile activity in the presence of 7.5 mM BDM, and were quiescent in the presence of 10 mM BDM. As seen in Figure 13, Northern blot analysis revealed that V, KCl, and BDM all down-regulated MHC- β and up-regulated SERCA2 mRNA levels. Of note, BDM treatment also appeared to decrease GAPDH mRNA levels for unknown reasons. Therefore, the mRNA levels encoding MHC- β and SERCA2 in this experiment were normalized to 18S rRNA levels. Of note, none of the arresting agents at the concentrations used in present study appeared to affect cell viability, as assessed by visual inspection of cultures for cell detachment, or loss of perchloric acid-precipitable DNA per well with time (Samarel, et al., manuscript in preparation). Quantitative analysis of 3 individual Northern blotting experiments is depicted in Figure 14, which demonstrates that each of the arresting agents increased SERCA2 mRNA levels, and decreased MHC- β

mRNA levels relative 18S rRNA. In summary, these results suggest that changes in mechanical activity rather than changes in $[Ca^{2+}]_i$ regulate SERCA2 mRNA levels.

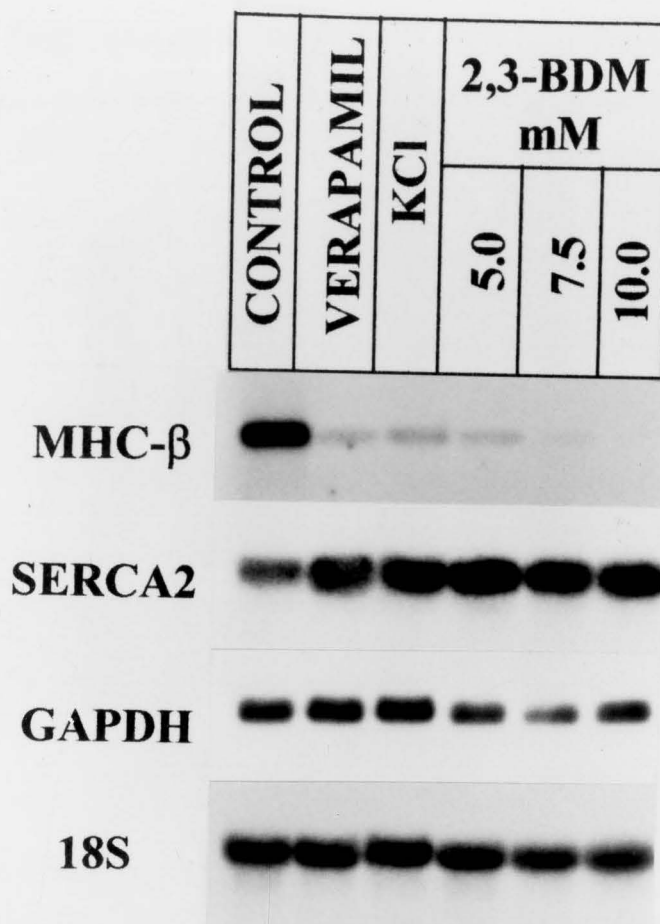


Figure 13: Northern blots for MHC- β and SERCA2 mRNA levels. Neonatal rat ventricular myocytes were maintained (48 h) in standard culture medium (Control) or medium supplemented with 10 μ M verapamil, 50 mM KCl or 5.0-10 mM 2,3-BDM. GAPDH mRNA and 18S rRNA levels are used to insure equal loading conditions.

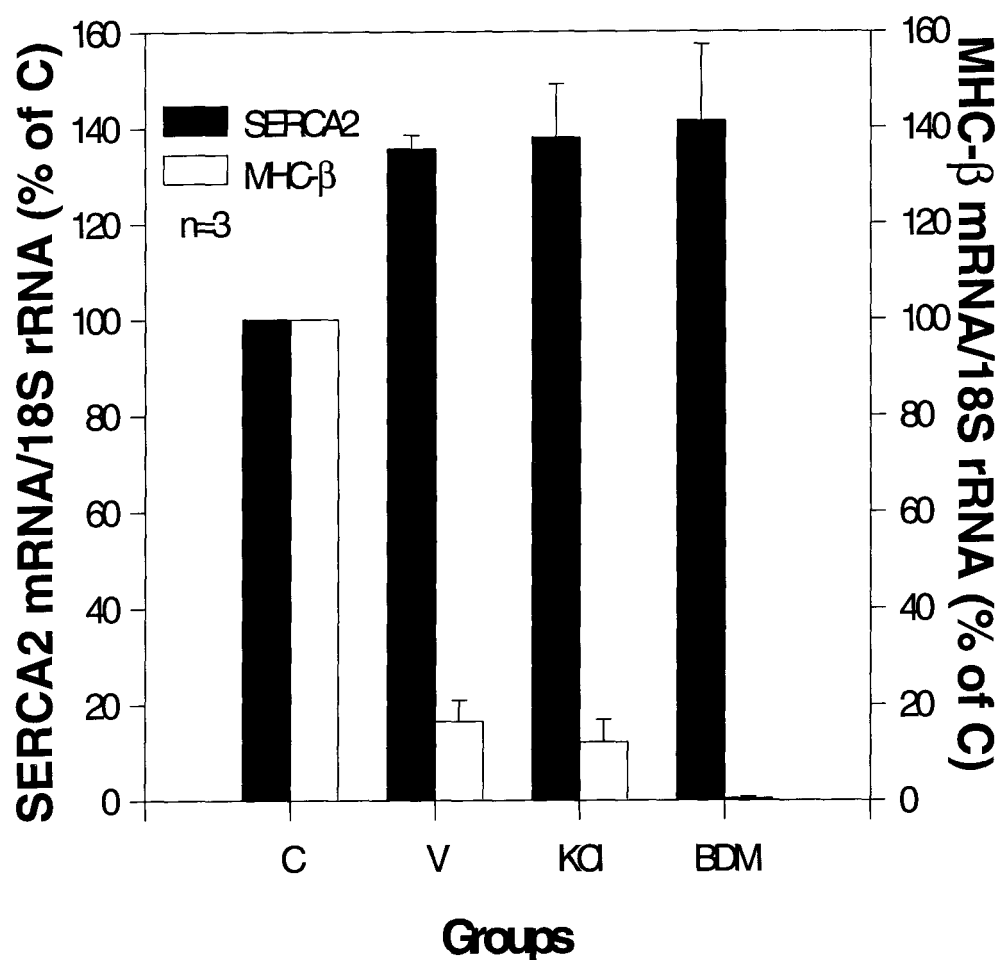


Figure 14: Quantitative analysis of SERCA2 and MHC- β mRNA levels in verapamil, KCl and BDM treated neonatal myocytes. Neonatal rat ventricular myocytes were maintained (48 h) in standard culture medium (Control) or medium supplemented with 10 μ M verapamil (V) or 50 mM KCl or 10 mM 2,3-BDM (BDM). SERCA2 and MHC- β mRNA levels were normalized with 18 S rRNA levels and Data were expressed as percent of control. Data are mean \pm SEM from 3 individual experiments.

3. Regulation of SERCA2 Gene Expression by Protein Kinase C

a). PMA produces myocyte hypertrophy. Addition of PMA (200 nM, 48 h) to the serum-free culture medium produced cardiac myocyte hypertrophy, as evidenced by a significant increase in total cellular protein and total protein/DNA ratio (Figure 15). These changes in protein accumulation were not dependent on contractile activity, as PMA increased total protein and protein/DNA ratio in both spontaneously contracting and verapamil-arrested myocytes. These data confirm previous results from this laboratory (Samarel et al., 1992), and indicate that PMA treatment stimulates cardiac myocyte hypertrophy in cultured neonatal rat ventricular myocytes.

b). SERCA2 mRNA levels are down-regulated during PMA-stimulated myocyte hypertrophy. As seen in Figure 16, PMA (200 nM, 48 h) increased MHC- β mRNA levels in both spontaneously contracting and verapamil-arrested neonatal myocytes. In contrast, PMA treatment markedly down-regulated SERCA2 mRNA levels in both contracting and verapamil-arrested cells. None of the mRNA levels were affected by treating either contracting or arrested myocytes with the inactive compound 4 α -phorbol (4 α P, 200 nM, 48 h). Quantitative analysis of 8-11 individual Northern blotting experiments is summarized in Figure 17. These results demonstrate that PMA significantly reduced SERCA2 mRNA levels by 57% and 42% in contracting and arrested cells, respectively. Of note, down-regulation of SERCA2 mRNA levels by PMA were not dependent on changes in contractile activity. This was evident from the lack of a significant interaction between the factors "PMA" and "Contraction" when the data in Figure 17 were analyzed by 2-way ANOVA.

The sensitivity of SERCA2 mRNA levels to PMA was also examined over a broad range (2-2000 nM) of concentrations. As seen in Figure 18, the half-maximal concentration for PMA-induced down-regulation of SERCA2 mRNA levels was approximately 50 nM. None of the PMA concentrations was associated with cytotoxicity,

as manifested by cell detachment or loss of DNA content over the 48 h observation period.

c). Regulation of SERCA2 mRNA levels by PKC activation. In all of the preceding experiments, myocytes were continuously exposed to varying concentrations of PMA for 48 h. Since prolonged incubation with PMA is known to cause activation, followed by down-regulation of some PKC isoenzymes (Johnson and Mochly-Rosen, 1995.), I further examined whether varying incubation time was associated with an increase, followed by a decrease in SERCA2 mRNA levels. As seen in Figure 19, exposure of myocytes to 200 nM PMA for 30 min - 72 h was not associated with a significant increase in SERCA2 mRNA levels as compared to untreated, control myocytes. Of note, SERCA2 mRNA levels in both control and PMA-treated myocytes decreased during the first several hours of maintenance culture (PMA > control), achieving stable level only after 12-24 h.

To test whether continuous exposure of myocytes to PMA was required for SERCA2 mRNA down-regulation over a 48 h period, I compared cells treated continuously for 48 h with PMA (200 nM) (P48 h), and cells that were treated with PMA for 0.5 h, followed by vigorously washing in HBSS to remove any traces of PMA, and maintenance culture for an additional 47.5 h in standard culture medium (P0.5h/C47.5h). As depicted in Figure 20, SERCA2 mRNA levels were markedly reduced in both P48 h and P0.5h/C47.5h cells, as compared to control myocytes maintained for the entire 48 h culture period in the absence of the drug.

To further ascertain the role of PKC-dependent signal transduction pathway(s) in the regulation of SERCA2 mRNA levels, neonatal rat ventricular myocytes were incubated with staurosporine (10 nM), a relatively selective inhibitor of PKC activity. The effects of staurosporine (10 nM), PMA (200 nM) and their combination on SERCA2 mRNA levels were quantitatively analyzed by Northern blotting analysis. As seen in Figure 21, staurosporine alone significantly ($P < 0.05$) increased SERCA2 mRNA levels as compared

to control myocytes. The PKC inhibitor also significantly ($P<0.05$) blocked the PMA-induced down-regulation of SERCA2 mRNA. In 2 similar experiments, the highly specific PKC inhibitor chelerythrine ($4\text{ }\mu\text{M}$) alone had no effect on SERCA2 mRNA levels, but completely blocked PMA induced down-regulation of SERCA2 mRNA levels (data not shown).

d). PMA reduces SERCA2 protein content in neonatal rat ventricular myocytes.

Whereas PKC activation by PMA produced significant down-regulation of SERCA2 mRNA levels, I examined whether the changes in transcript levels were also accompanied by changes in the relative concentrations of SR Ca ATPase protein, as determined by Western blotting using a highly specific, polyclonal antibody to SR Ca ATPase. Neonatal rat ventricular myocytes were incubated with or without PMA (200 nM) for 72 h, and equal amounts of total cellular protein were separated by SDS-PAGE and Western blotting. The SERCA2 antiserum detected a prominent, 110 kDa . band consistent with the apparent molecular weight of rat SR Ca ATPase (Figure 22). Quantitative analysis of SERCA2 protein levels in 8 separate Western blotting experiments is depicted in Figure 23. PMA treatment for 72 h significantly reduced SERCA2 protein levels to 47% of the control cells ($n=8$ experiments, $P<0.0001$).

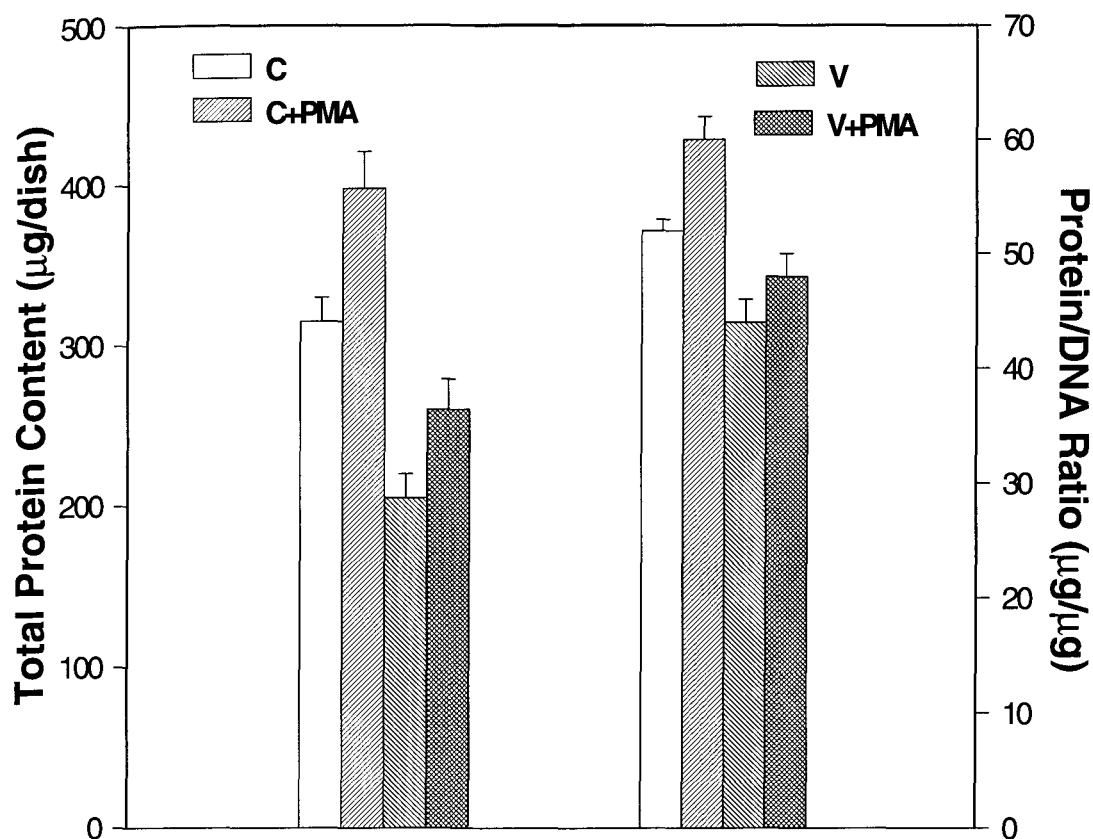


Figure 15: Effect of PMA on growth of neonatal rat heart cells. Neonatal rat ventricular myocytes were maintained (48h) in standard culture medium (C) or medium supplemented with 10 μM verapamil (V), or 200 nM phorbol 12-myristate, 13-acetate (PMA) or their combination. Data are Mean±SEM of duplicate wells of each treatment group from 6-17 different cell isolations. Effects of factors contraction (“C”), PMA, and their interaction (“C-PMA”) on these biochemical parameters were analyzed by 2-way ANOVA. The results indicate that both PMA and contraction have significant effects on these biochemical parameters. However, there is no interaction between the factors “PMA” and “C”, indicating that PMA increased total protein and protein/DNA ratio independent of the contractile activity of the myocytes.

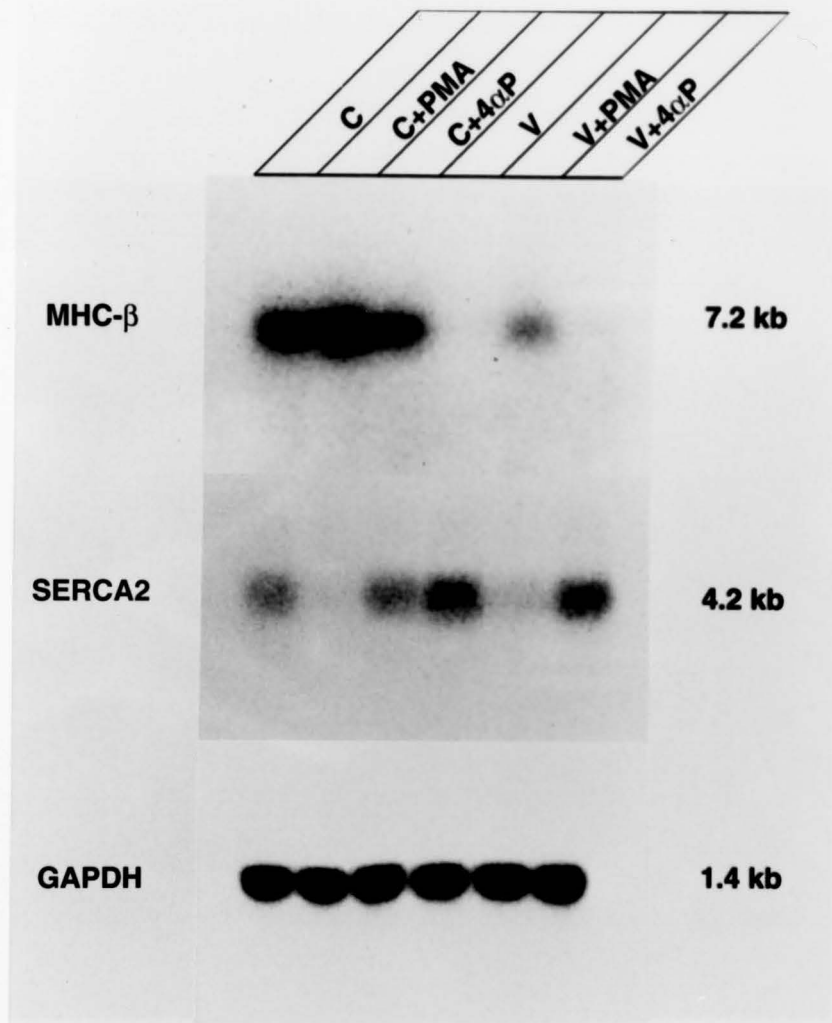


Figure 16: PMA alters SERCA2 mRNA levels. Spontaneously contracting (C) neonatal myocytes were maintained (48 h) in serum-free medium, or growth medium supplemented with 10 μ M verapamil (V) to produce contractile arrest. The PKC activator phorbol 12-myristate 13-acetate (C+PMA, 200 nM) or the inactive phorbol 4- α -phorbol (C+4 α P, 200 nM) were also added to the culture medium of contracting or V-arrested cells. Numbers to the right of each autoradiogram are the size (in kilobases) of each mRNA transcript.

Figure 16: PMA alters SERCA2 mRNA levels. Spontaneously contracting (C) neonatal myocytes were maintained (48 h) in serum-free medium, or growth medium supplemented with 10 μ M verapamil (V) to produce contractile arrest. The PKC activator phorbol 12-myristate 13-acetate (C+PMA, 200 nM) or the inactive phorbol 4- α -phorbol (C+4 α P, 200 nM) were also added to the culture medium of contracting or V-arrested cells. Numbers to the right of each autoradiogram are the size (in kilobases) of each mRNA transcript.

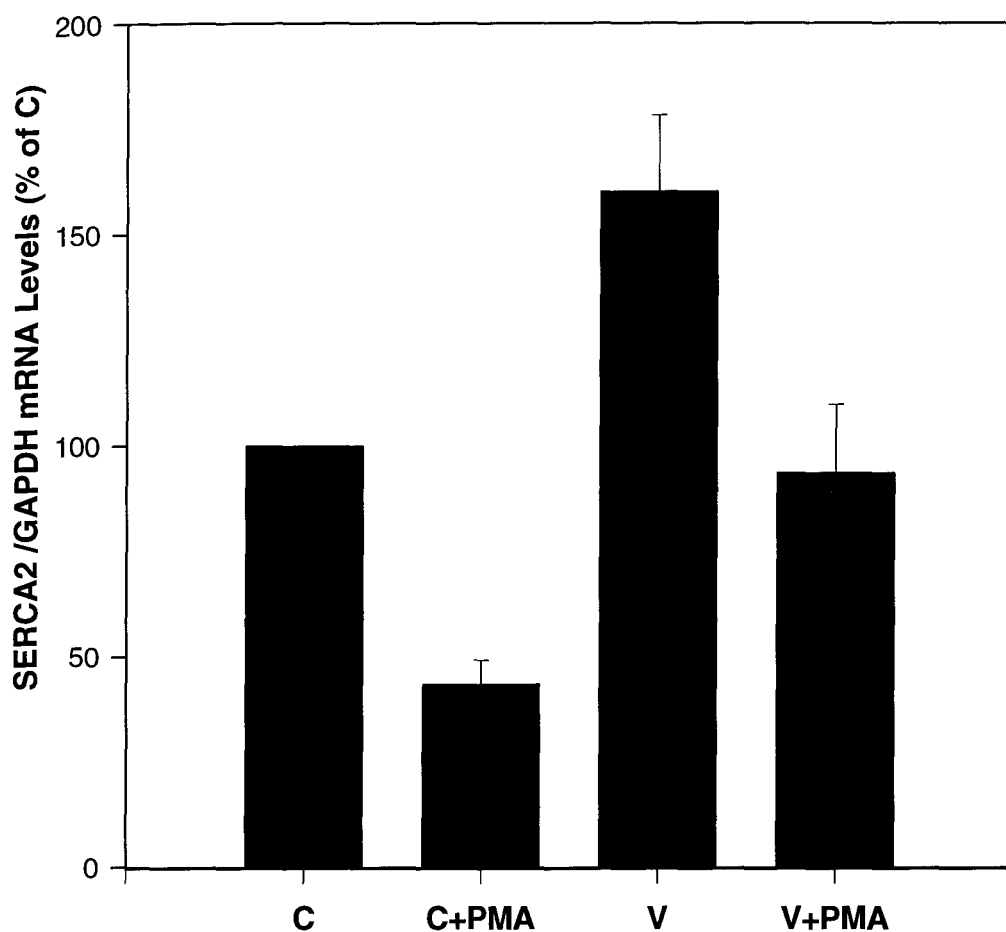


Figure 17: PMA induced down-regulation of SERCA2 mRNA levels in spontaneously contracting (C) and verapamil-arrested (V) myocytes. Data were obtained from 8-11 individual Northern blotting experiments. The intensities of SERCA2 mRNA were normalized to the intensities of GAPDH mRNA and plotted as the percentage of the value of C cells. Data were analyzed by 2-way ANOVA, which indicated that both contractile activity and PMA significantly altered SERCA2 mRNA levels. However, there was no interaction between the factors “PMA” and “C”, indicating that PMA decreased SERCA2 mRNA levels independent of the contractile activity of the myocytes.

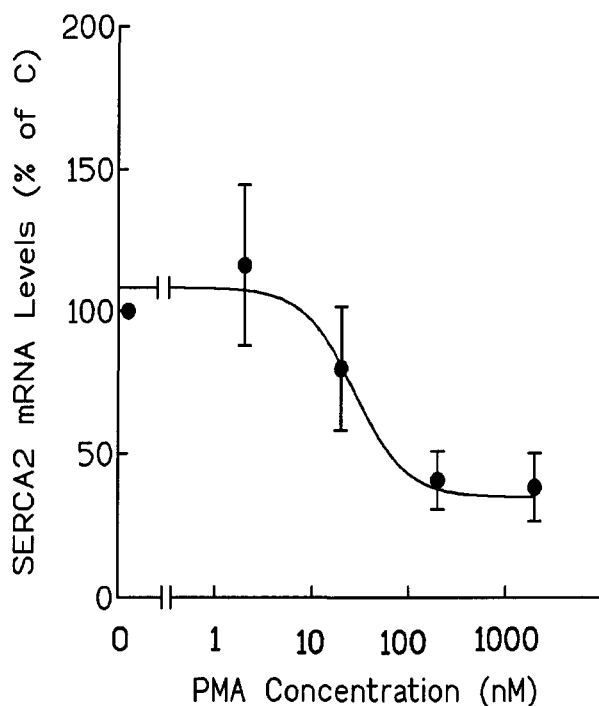


Figure 18: Dose-dependence of SERCA2 mRNA levels to PMA treatment. Neonatal myocytes were maintained in serum-free growth medium containing 0-2000 nM PMA for 48 h. Total RNA was isolated and the Northern blots were sequentially hybridized with ^{32}P -labeled SERCA2 and GAPDH cDNA probes. Autoradiograms were scanned by laser densitometry and the ratio of SERCA2 /GAPDH mRNA band intensity was plotted as the percentage of the value in control (0 nM, C) cells. Data are means \pm SEM from 4 individual experiments.

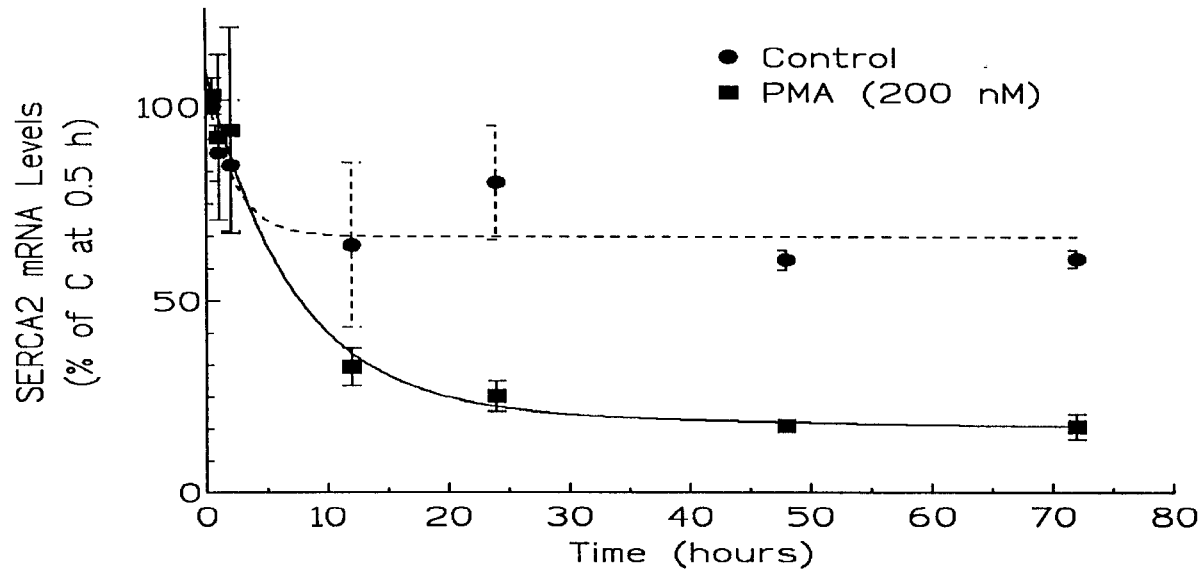


Figure 19: Time-dependent changes in SERCA2 mRNA levels in control and PMA-treated cells. Total RNA was isolated at 0.5, 1, 2, 12, 24, 48 and 72 h of maintenance culture in serum-free growth medium in the presence or absence of PMA (200 nM). SERCA2 mRNA levels were analyzed by Northern blotting and quantified relative to the level of GAPDH mRNA. Data were plotted as the percentage of the SERCA2/GAPDH mRNA ratio of control cells at 0.5 h. Data are the mean \pm SEM of 3 experiments.



Figure 20: Prolonged exposure to PMA is not necessary for SERCA2 mRNA down-regulation. Neonatal rat ventricular myocytes were maintained in serum-free growth medium for 48 h (C), growth medium containing 200 nM PMA for 48 h (P48h) or growth medium containing 200 nM for 0.5 h followed by vigorous washing and maintenance culture for an additional 47.5 h in standard culture medium (P 0.5h/C47.5h). Total RNA (10 μ g) was subjected to Northern blotting and sequentially hybridized with 32 P-labeled cDNA probes specific for SERCA2 and GAPDH mRNAs.

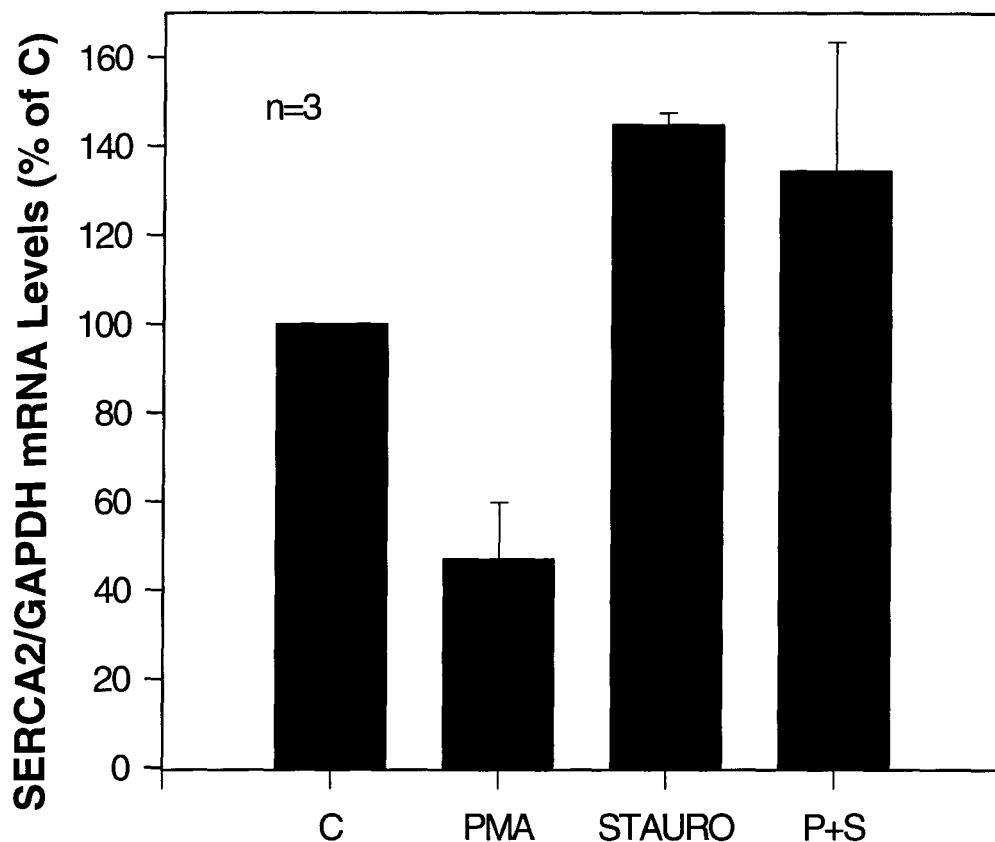


Figure 21: Effects of PMA and staurosporine on SERCA2 mRNA levels. Neonatal rat ventricular myocytes were maintained for 48 h in serum-free medium (C), medium containing 200 nM PMA (PMA), medium containing 10 nM staurosporine (STAURO) or medium containing both 200 nM PMA and 10 nM STAURO (P+S). The intensities of SERCA2 band on the Northern blots were normalized to the intensity of GAPDH mRNA levels and plotted as percent of the value in C cells. Data are the means \pm SEM of 3 experiments.

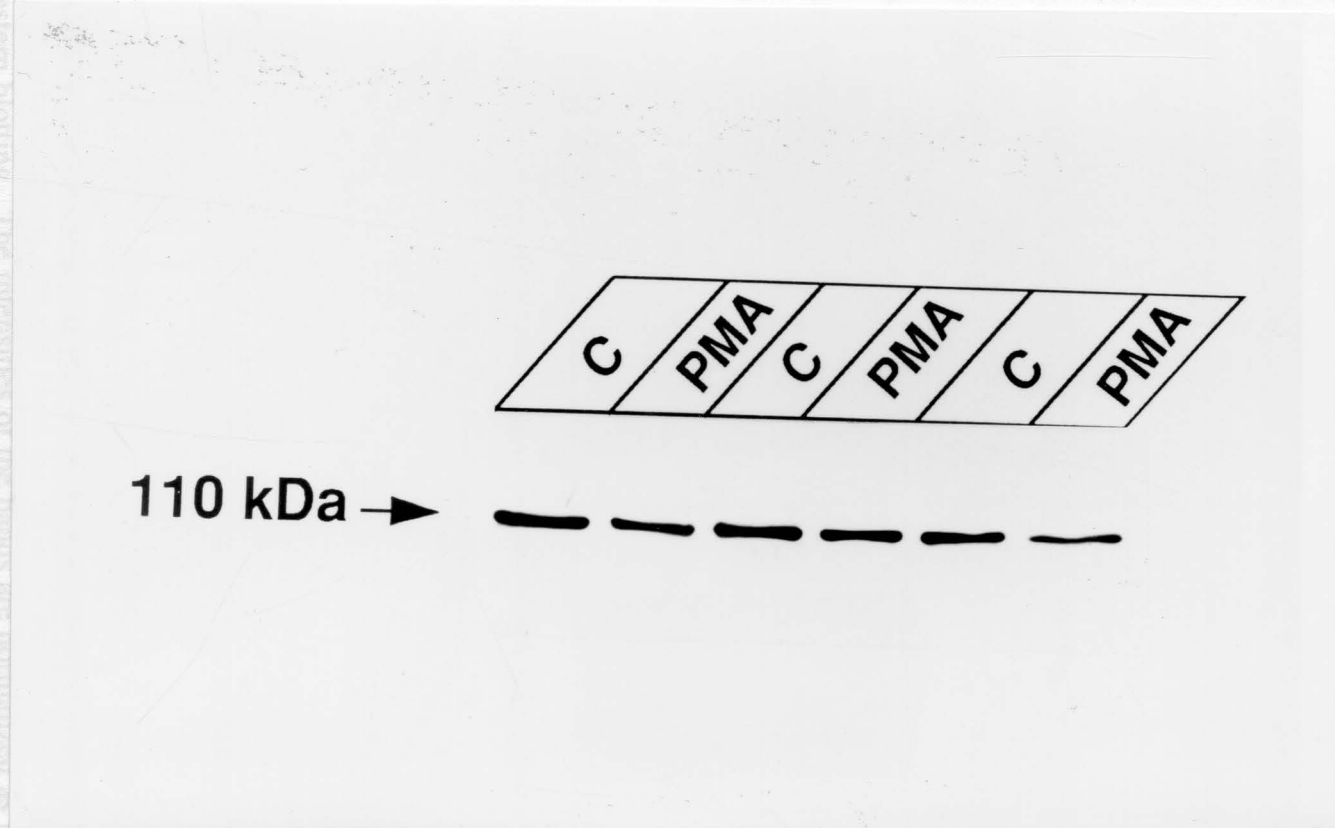


Figure 22: Western blot for SR Ca ATPase protein levels. Neonatal rat ventricular myocytes were maintained in serum-free growth medium in the presence (PMA) or absence (C) of 200 nM PMA for 72 h. The cells were harvested and equal amounts of cellular protein (40µg) were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose by electroblotting, and probed with specific antiserum to rat SERCA2. The bands were detected by ECL technique. Depicted in the figure are Western blots from 3 different experiments. The arrow on the left indicates the apparent molecular weight in kilodotons.

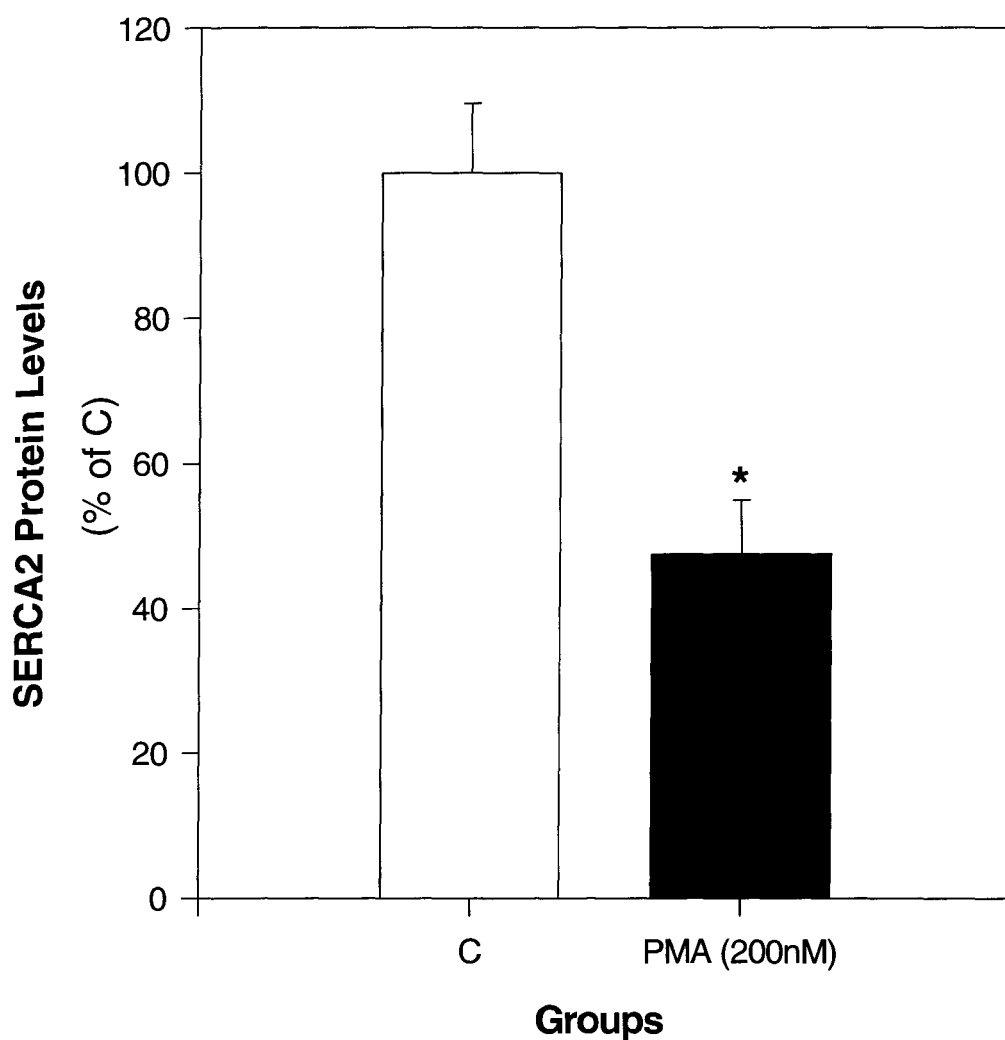


Figure 23: PMA induced down-regulation of SERCA2 protein levels. Neonatal rat ventricular myocytes were maintained in serum-free growth medium in the presence (PMA) or absence (C) of 200 nM PMA for 72 h. SERCA2 protein levels were analyzed by Western blotting. The intensities of the bands are normalized as percent of that in C cells. Data are the mean \pm SEM from 8 individual experiments.

C. TRANSCRIPTIONAL VS. POST-TRANSCRIPTIONAL REGULATION OF SERCA2 GENE EXPRESSION

1. Transcriptional Regulation of SERCA2 Gene Expression.

a). PCR Cloning of the SERCA2 Promoter. I used a proximal promoter-reporter gene construct to analyze the effects of verapamil and PMA on the rate of SERCA2 gene transcription. A PCR-based cloning technique was used to obtain a DNA fragment from the 5' untranslated region of rat SERCA2 gene. The PCR product was analyzed on an agarose gel, which revealed a single amplification product corresponding to the expected length of the fragment based upon the previously published sequence of the rat gene (Figure 24). I subsequently sequenced this 616 bp PCR product in both directions using the dideoxy chain termination method. My sequence was identical to the promoter sequence described by Hartong et al (1991) except for 4 base substitutions (Figure 25). This 616 bp DNA fragment was successfully subcloned into the promoterless luciferase expression plasmid (pluc), yielding a 6.4 kb promoter-reporter gene construct termed pSERCA-luc (Figure 26).

b). PMA changes the transcription rate of SERCA2 gene expression. A transient transfection assay was then used to determine whether verapamil or PMA altered the rate of SERCA2 gene transcription. I transfected cultured neonatal rat ventricular myocytes with pSERCA-luc and a constitutively expressed reporter plasmid (pRSVlacZ). pRSVlacZ, an expression plasmid containing the bacterial lacZ gene encoding β -galactosidase driven by the Rous sarcoma virus promoter, was used to control for

differences in transfection efficiency. Following lipofection, the cells were maintained (48 h) in standard growth medium, or growth medium containing verapamil (10 μ M), PMA (200 nM) or their combination. Luciferase and β -galactosidase (β -gal) activities were measured immediately after harvesting the cells. Both the SERCA2 promoter-reporter gene construct (pSERCA-luc) and the reference plasmid (pRSVlacZ) were highly expressed in cultured myocytes. Luciferase activity in the cells transfected with pSERCA-luc was 2000-4000 times greater than that in the cells transfected with the promoterless pluc plasmid, indicating that pSERCA-luc has very high promoter activity in these cells. As shown in Figure 27, verapamil treatment had no effect on normalized luciferase activity. In contrast, PMA treatment significantly (n=9 experiments; $P < 0.05$) increased normalized luciferase activities in both contracting and verapamil-arrested cells (2.2 ± 0.2 and 1.6 ± 0.1 fold, respectively). These results indicated that the verapamil- and PMA-induced changes in SERCA2 mRNA levels were not primarily regulated at the transcriptional level.

2. Effects of Verapamil and PMA on SERCA2 mRNA Stability.

Act-D mRNA stability assays were then used to determine whether verapamil or PMA affected the stability of SERCA2 mRNA. The neonatal rat ventricular myocytes were maintained in growth medium in the absence or presence of verapamil (10 μ M) or PMA (200 nM) for 24 h. The cultures were then exposed to the RNA synthesis inhibitor actinomycin-D (act-D, 5 μ g/ml) for an additional 0, 12 and 24 h. As seen in Figure 28, SERCA2 mRNA levels in control myocytes were only reduced by approximately 20%

after 24 h exposure to Act-D, indicating that previously transcribed SERCA2 mRNA was relatively stable in these culture. Nonlinear regression analysis of these data revealed an apparent half-life of SERCA2 mRNA in control myocytes of approximately 63 hours. In contrast, SERCA2 mRNA levels in PMA-treated myocytes were reduced by over 50% within 24 h of Act-D exposure, corresponding to an apparent half-life of approximately 23 hours. Using a similar analysis, SERCA2 mRNA levels in verapamil treated cells appeared very stable over the time range of my observation ($t_{1/2} > 600$ h).

I interpret these results as indicating that the verapamil induced up-regulation of SERCA2 gene expression in cardiac myocytes results from the stabilization of SERCA2 mRNA. On the other hand, down-regulation of SERCA2 gene expression by PMA was at least in part the result of a significant reduction in the half-life of this transcript.

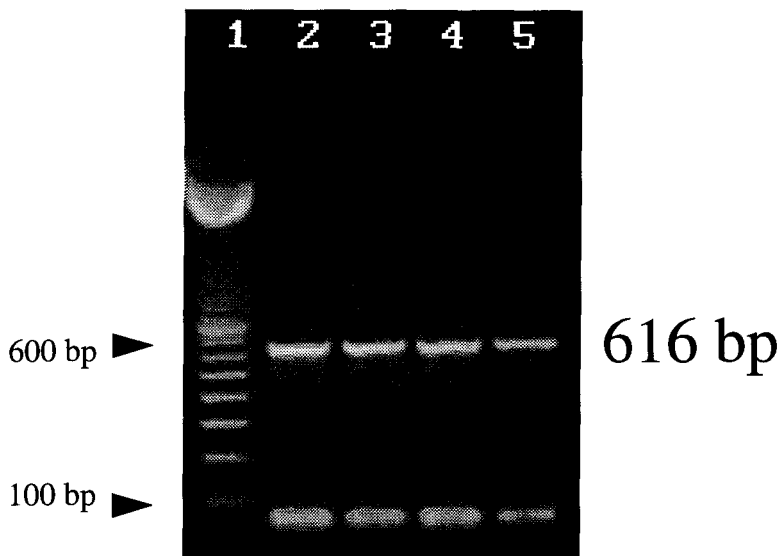


Figure 24: The 616 bp PCR product. To estimate the size of the PCR product, the PCR reaction mixtures were loaded onto a 1.5 % agarose gel. Line 1 is a 100 bp ladder DNA molecular weight standard, Line 2-5 are portions of the PCR reaction mixture. Numbers to the left indicate the size the DNA fragments within the standard. Number to the right indicates the estimated molecular weight of the PCR product.

```

*** INPUT INFORMATION ***

NO. FILE NAME      BP      A      C      G      T/U  SEQUENCE POSITION
1  RATSRCOA.DNA    616    83    232   231    70      1 -    616
2  MING.SEQ        616    85    230   230    71      1 -    616

*** SEQUENCE LIST ***

RATSRCOA.DNA      10      20      30      40      50
MING.SEQ          4  TTCCTCCCCT  TGGTTGCTGA  GGGGGGCTCT  GAAGGAGACA  TTAGGATGCA  53
                   1  *****  *****  *****  *****  *****  50

RATSRCOA.DNA      60      70      80      90     100
MING.SEQ          54  GAGCACGGCT  CGCGCTGCC  AGGGCGCGGA  GGCAAGCCAA  GGACACCAGT  103
                   51  *****A  *****  *****  *****  *****  100

RATSRCOA.DNA     110     120     130     140     150
MING.SEQ        104  CCTCGCGCG  CTCGTCGCC  AGCGTCCGG  CGCTCCCTG  CCACACGCG  153
                   101  *****  *****  *****  *****  *****  150

RATSRCOA.DNA     160     170     180     190     200
MING.SEQ        154  GCCCCAGGA  GTCCCCCTC  AGCGGCGGG  CCCCAGCAC  AGAGCGCCA  203
                   151  *****  *****  *****  *T*****  *****  200

RATSRCOA.DNA     210     220     230     240     250
MING.SEQ        204  CCCGTGAGCT  CCGGATCTA  TTCCTGCAC  CGGACGGAAT  GGGAAAGCCG  253
                   201  *****  *****  *****  *****  *****  250

RATSRCOA.DNA     260     270     280     290     300
MING.SEQ        254  CGACCGCTA  AGGTCGGGT  CGCCCTCCG  CGCCCGGGC  CGCAGCGAGC  303
                   251  *****  *****  *****  *****  *****  300

RATSRCOA.DNA     310     320     330     340     350
MING.SEQ        304  ACACGAGGA  CGCGGAGGT  GCCTGCGCG  GCACACGCG  GCGGCTCGA  353
                   301  *****  *****  *****  AT*****  *****  350

RATSRCOA.DNA     360     370     380     390     400
MING.SEQ        354  TCCGGTTAC  TGGGGGCGG  GCGCGGGAG  AGGCGGGGC  TGCCGGCAGC  403
                   351  *****  *****  *****  *****  *****  400

RATSRCOA.DNA     410     420     430     440     450
MING.SEQ        404  GTGGGCGCG  ACGCGCCCG  GGAGGGCGC  GGGGAGGGG  GCGGGGCGC  453
                   401  *****  *****  *****  *****  *****  450

RATSRCOA.DNA     460     470     480     490     500
MING.SEQ        454  GCCGCCGCG  CCGCGCTGG  CTCTCTCGG  CAATGAGCG  CGTCCACATG  503
                   451  *****  *****  *****  *****  *****  500

RATSRCOA.DNA     510     520     530     540     550
MING.SEQ        504  CCCGGCGCG  GCGAGAGGG  AGGCAGCGC  GGATAAATG  TATTAGAGCA  553
                   501  *****  *****  *****  *****  *****  550

RATSRCOA.DNA     560     570     580     590     600
MING.SEQ        554  GCCTCCGCG  AGCCGTCCC  GACGCCACG  CCCGTCTCT  CCGCCGCGT  603
                   551  *****  *****  *****  *****  *****  600

RATSRCOA.DNA     610     620     630     640     650
MING.SEQ        604  TTCCTCCGC  GCTGTC....  .....  .....  .....  653
                   601  *****  *****  .....  .....  .....  650

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Figure 25: Comparison of the PCR product sequence with SERCA2 promoter sequence. MING.SEQ=PCR product sequence, RATSRCOA.DNA =SERCA2 promoter sequence. * represents the identical base.

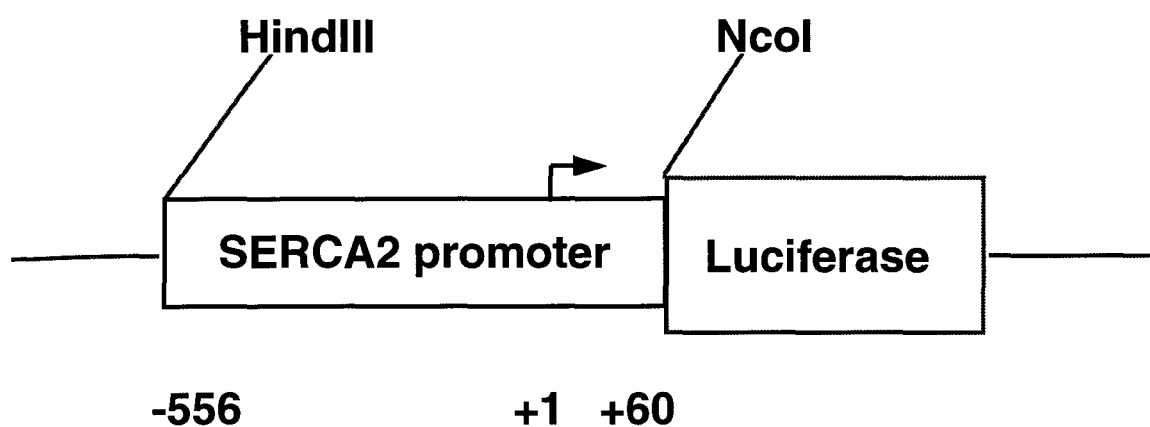


Figure 26: SERCA2 promoter and promoterless luciferase gene construct plasmid. HindIII and NcoI are the unique restriction enzyme sites in the promoter and plasmid. The numbers are bases relative to the position of the SERCA2 gene transcription start site. +1 is the transcription start site for SERCA2 gene.

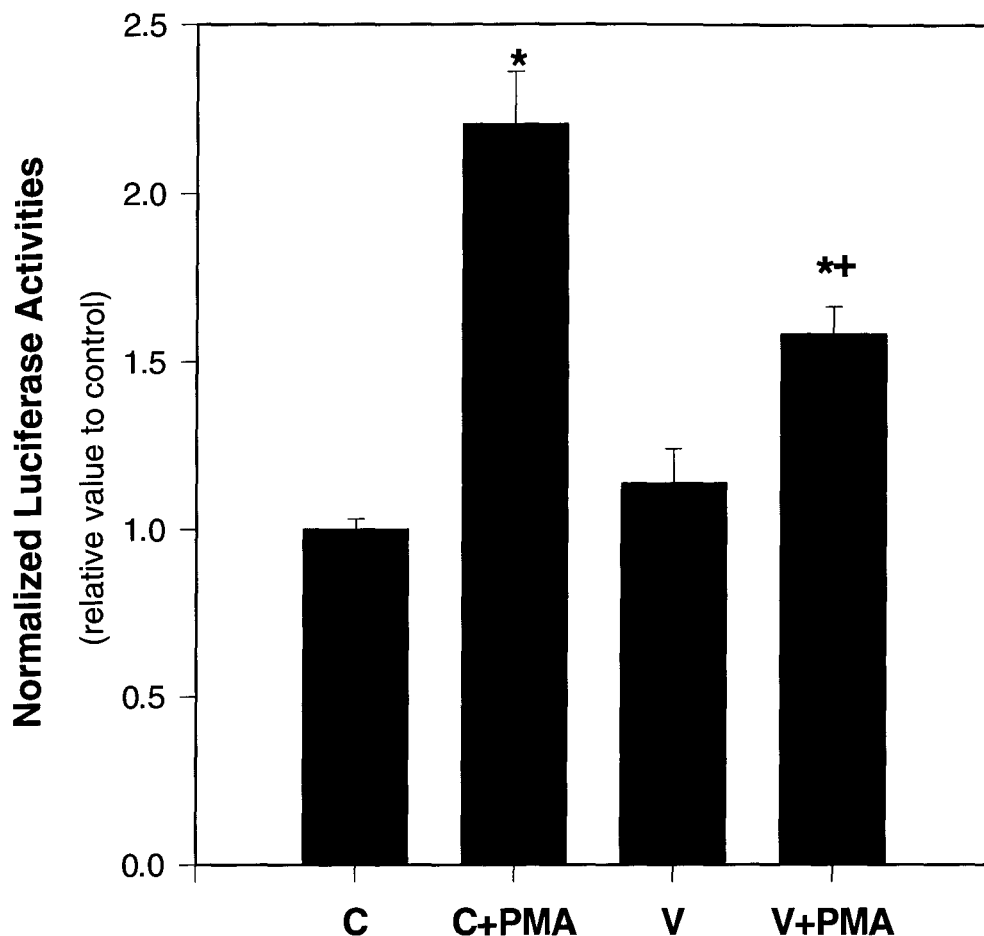


Figure 27: PMA increased SERCA2 mRNA transcription rate in spontaneously contracting (C) and verapamil arrested (V) cells. Neonatal rat ventricular myocytes were transfected with the promoter-reporter gene construct plasmid, pSERCA-luc, and the control plasmid, pRSVlacZ. The cell cultures were then maintained in growth medium in the absence or presence of 10 μ M verapamil or 200 nM PMA for an additional 48 h. The cells were harvested for the measurement of luciferase and β -gal activities. Luciferase activity was normalized to β -gal activity and expressed as percent of control (contracting) cells. Data are Mean \pm SEM from 9 experiments. Friedman repeated measures analysis of variance on ranks test followed by Student-Newman-Keuls test were used for statistical comparisons of the multiple groups. * $P < 0.05$ vs C, + $P < 0.05$ vs V.

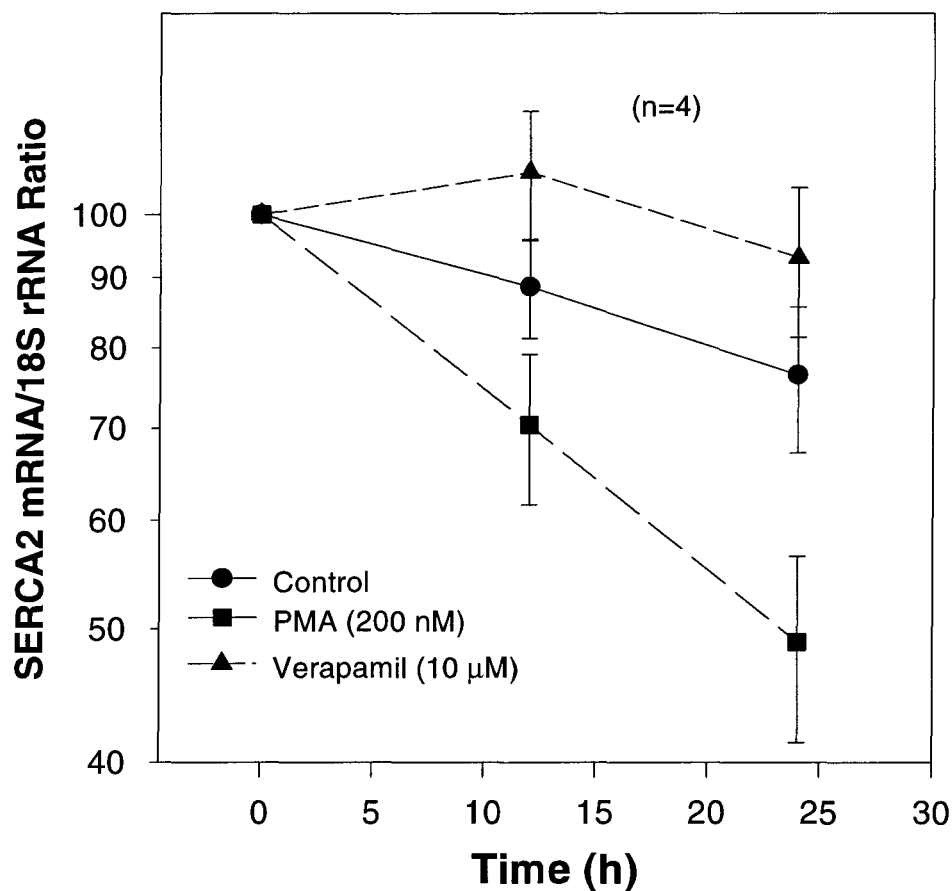


Figure 28: Effects of verapamil and PMA on SERCA2 mRNA stability. The relative stability of SERCA2 mRNA in control, verapamil and PMA-treated cells was assessed following treatment of myocytes with actinomycin D (Act-D). Neonatal myocytes were maintained in serum-free growth medium (Control), medium supplemented with 200 nM PMA (PMA) or medium containing 10 μM verapamil (Verapamil) for 24 h. Act-D (5 μg/ml) was then added, and the cells maintained for an additional 0, 12, or 24 h at which times total RNA was isolated and analyzed by Northern blotting with probes specific for SERCA2 mRNA and 18S rRNA. Data are normalized to the ratio of SERCA2 mRNA/18S rRNA observed at time 0 of control, PMA or verapamil treated cells, respectively, and plotted as a function of time. Data are the means±SEM of 4 different experiments.

CHAPTER V

DISCUSSION

A. DO CHANGES IN SERCA2 GENE EXPRESSION AFFECT CARDIAC PERFORMANCE DURING LVH PROGRESSION?

1. Adult Rat Abdominal AoC Model and Hemodynamic Measurements.

In my dissertation studies, I used a rat abdominal AoC model to examine whether changes in SERCA2 gene expression affected cardiac performance during LVH progression. This model closely mimics chronic pressure overload hypertrophy in humans since coarctation of the abdominal aorta of the juvenile rat produces a relatively gradual onset of sustained hypertension, thus minimizing acute myocardial injury. My results demonstrate that abdominal AoC produced sustained hypertension and LVH, as evidenced by a marked elevation in the LV/body weight ratio. In agreement with others (Mercadier et al., 1991), the present study also shows that pressure overload LV was

accompanied by a switch in MHC isoenzyme predominance (i.e. from MHC- α to MHC- β) which is characteristic of the LVH phenotype.

The current literature does not provide direct evidence for a causative role for SERCA2 down-regulation in the diastolic dysfunction that accompanies pressure overload hypertrophy, due to the lack of serial hemodynamic measurements correlated with alterations in SERCA2 gene expression. This gap in our knowledge was addressed in the present study, in which *in vivo* hemodynamic measurements of cardiac function were obtained during the progression of LVH. Systolic function was evaluated by $+dP/dt_{\max}$, and CI. Maximal $-dP/dt$ ($-dP/dt_{\max}$), $(-dP/dt_{\max})/MAP$, and τ were used as indices of cardiac relaxation. LVEDP was also used as an index of overall cardiac performance. As seen in Table 5, $+dP/dt_{\max}$, and CI were not significantly altered in either 8 wk and 16 wk banded animals, indicating that isovolumic systolic function was relatively normal despite increased afterload and LVH. In contrast, banded rats demonstrated abnormalities in diastolic function, especially at 16 weeks. The $-dP/dt_{\max}$ was reduced in both 8 and 16 wk banded rats; however, statistically significant differences were not reached. Since $-dP/dt$ is dependent on the peak aortic pressure rather than on the rate of ventricular pressure decline (Lorrell, 1991), $(-dP/dt_{\max})/MAP$ was used to minimize the effect of afterload on $-dP/dt$ as previously described (Burgess et al., 1995). In the present study, significantly reduced $(-dP/dt_{\max})/MAP$ was an indication of slowed cardiac relaxation in both 8 and 16 wk banded rats (Table 5). Furthermore, one of

the most accurate measures of relaxation *in vivo* is the time constant for isovolumic relaxation (τ). It has been widely used in many studies for a review see Gilbert and Glantz, 1989). Because the time course for isovolumic pressure decline begins after aortic valve closure, τ is relatively independent of peak aortic pressure and peripheral factors which determine the impedance to ventricular ejection (Weiss et al, 1976). In the present study, prolongation of τ indicated impaired cardiac relaxation occurred in 16 wk pressure overloaded hearts (Table 5). However, the τ values in 8 wk banded rats were not statistically different from that in sham rats due to the large variability between experimental animals. Nevertheless, the τ values in sham rats were comparable with other studies (Litwin et al., 1990). In addition, LVEDP was normal in 8 wk banded rats, but was significantly ($P < 0.01$) elevated in 16 wk banded animal. These results indicate a gradual development of pressure overload hypertrophy from compensated LVH (8 wk banded rats with normal LVEDP) to a transitional phase between compensated LVH and CHF (16 wk banded rats with markedly elevated LVEDP). As seen in Figure 4, τ and LVEDP were not changed by acute constriction of the abdominal aorta. These data indicate that τ and LVEDP are insensitive to changes in afterload per se, but reflect intrinsic changes in cardiac structure or function that developed over time.

2. Could the Decline in Diastolic Function During Pressure Overload LVH Result From Decreased SERCA2 Gene Expression?

Down-regulation of SERCA2 mRNA levels has been reported in pressure overload hypertrophied and failing hearts in both patients and experimental animal models (Komuro et al., 1989, Arai et al., 1993, Hasefuss et al., 1994). It has been suggested that down-regulation of SERCA2 gene expression may be the most important contributor to the relaxation abnormality during pressure overload hypertrophy and CHF (Schwartz et al, 1992). However, a cause-and-effect relationship between altered mechanical properties and SERCA2 gene expression has not been clearly established. In the present study, I examined both SERCA2 mRNA and protein levels in two different stages (compensated LVH and the transitional phase between compensated LVH and HF) of chronic pressure overload hypertrophy in the rat. The results show that SERCA2 mRNA levels were markedly down-regulated in compensated LVH (8 wk banded rats), and continued to decline as LV cardiac performance deteriorated. Importantly, reduced SERCA2 gene expression occurred in the absence of hemodynamic changes consistent with severe cardiac decompensation and CHF. These data support the notion of Feldman et al. (1993) that reduced SERCA2 mRNA levels may be a predictive marker of the transition from compensatory hypertrophy to HF in the pressure overloaded heart. However, they also indicate that time is a very important factor. Nevertheless, SERCA2 protein levels were not reduced in 8 wk banded rats, despite a prolongation in myocardial relaxation. These data argue against a significant contribution of SERCA2 down-regulation to the subtle alterations in diastolic function observed in 8 wk banded rats. Despite a normal concentration of SR Ca ATPase protein in 8 wk banded animals, the

reduction in the number of SR Ca^{2+} pumps in 16 wk hypertensive animals did correlate with the marked increase in LVEDP and the significant prolongation of τ . These data indicate down-regulation of the SERCA2 gene may indeed play an important role in the progressive deterioration of LV performance over time.

The disparity between SERCA2 mRNA and protein levels at 8 and 16 weeks suggests that this integral membrane protein possesses a very long half-life in the hypertrophied LV myocardium, and the effects of decreased SERCA2 synthesis required many weeks to become manifest. Alternatively, it is conceivable that an increase in the efficiency of protein synthesis that accompanies pressure overload (Nagai et al., 1987) could partially compensate for a reduction in the steady-state level of SERCA2 mRNA, producing no significant change in the amount of SERCA2 protein synthesis until levels of this transcript were markedly reduced. It is also conceivable that reduced SR Ca^{2+} pump activity (due to changes in the amount or phosphorylation state of phospholamban, ATPase availability or other regulatory factors) rather than reduced numbers of SR Ca^{2+} pumps contributed to slowed relaxation in 8 wk banded animals. Future studies are required to evaluate these possibilities.

3. Could The Decline in Diastolic Function During Pressure Overload LVH Result From Changes in the cardiac ECM?

As discussed by Tsutsui et al. (1993), the velocity of relaxation is dependent upon both intrinsic restoring forces (i.e. the potential energy that is produced by the deformation occurring during contraction and that is released during relaxation) and the rate of myocardial inactivation. It has been suggested that ECM remodeling and interstitial fibrosis may alter the elastic recoil properties of hypertrophied ventricular tissue (Weber et al., 1993). Thus changes in the concentration or composition of ECM components may play a role in altered cardiac relaxation in cardiac hypertrophy and failure. In fact, increased myocardial passive stiffness can precede the development of systolic dysfunction during pressure-overload-induced LVH (Weber et al., 1993). In the experimental model of pressure overload used in this study (Eleftheriades et al., 1993), increased Type I collagen gene expression, replacement fibrosis and increased myocardial stiffness are prominent features associated with LV diastolic dysfunction, despite relatively normal isovolumic systolic performance 16 wk after AoC. Increased LV total collagen content, and a decrease in the ratio of Type III to Type I collagen gene expression were also observed in this model (Eleftheriades et al., 1993). Furthermore, Burgess et al. (1995) recently demonstrated that Sprague-Dawley rats with unilateral renal ischemia and 10 wk of sustained hypertension developed a similar degree of slowed myocardial relaxation as was observed in the present study, again without impairment of

LV systolic function. It is interesting to speculate whether these animals would have eventually developed LV dilatation and overt signs of systolic dysfunction, as was observed in subsets of 18-24 mo old SHR (Boluyt et al., 1994; Conrad et al., 1995), Fisher 344 rats 8 months after renal artery constriction (Capasso et al., 1990) and Wistar rats 20 wk after ascending aortic banding (Feldman et al., 1993). Nevertheless, the rather subtle alterations in myocardial relaxation observed in our 8 wk hypertensive animals were temporally related only to the presence of LVH and disproportionate collagen accumulation, and not to changes in the number of SR Ca^{2+} pumps or changes in myosin composition. Conversely, worsening cardiac function at 16 wk was not associated with a further increase in LV mass or fibrillar collagen content, suggesting that the progressive impairment in diastolic function over time was caused by additional factors. As discussed above, down-regulation of SERCA2 gene expression may have contributed to the further deterioration of LV diastolic function, even though it was not responsible for the impaired cardiac relaxation observed in 8 wk banded animals.

4. Could The Decline in Diastolic Function During Pressure Overload LVH Result From The Myosin Isoenzyme Switch?

Myocardial inactivation is determined by the biochemical processes leading to the detachment of crossbridges, the reduction in a rate of crossbridge cycling, and a decline in $[\text{Ca}^{2+}]_i$ (Tsutsui et al., 1993). A switch in MHC isoenzymes has been shown to affect the

rate of crossbridge detachment and the overall rate of crossbridge cycling, thereby reducing the rate of inactivation (Brutsaert and Sys, 1989). Therefore, the decline in diastolic function during pressure overload LVH may be related to the change in the expression of MHC- β in cardiac myocytes. However, I observed a reduction in the rate of myocardial relaxation (as assessed by the normalized $-dP/dt$ and possibly τ) prior to a major change in the relative concentration of MHC- β . MHC- β was the predominant MHC isoenzyme expressed in LV myocytes of 16 wk banded animals. These results indicate that a switch in MHC isoenzymes was not responsible for the relaxation abnormalities observed in 8 wk banded animals. However, the isoenzyme switch may have contributed to the deterioration in cardiac performance in 16 wk banded animals.

5. Conclusions

Chronic pressure overload produced LVH and a progressive deterioration in LV function. The slowed cardiac relaxation in compensated LVH (8 wk banded rats) did not appear to result from down-regulation of SERCA2 gene expression. Other factors such as remodeling of the cardiac ECM, or a decrease in SR Ca^{2+} ATPase activity (without a reduction in the number of SR Ca pumps) may be responsible for the relaxation abnormality observed during the compensated phase of pressure overload LVH. Reduced SERCA2 mRNA levels preceded the onset of overt cardiac dysfunction, indicating that down-regulation of SERCA2 gene expression may be a useful marker to predict the

ultimate development of cardiac dysfunction. Furthermore, decreased SERCA2 mRNA and protein levels in 16 wk banded rats suggest that down-regulation of SERCA2 gene plays an important role in the deterioration of LV function during the progression of pressure overload LVH. Therefore, it is very important to determine the intracellular mechanisms responsible for SERCA2 down-regulation during LVH progression, and to elucidate the specific molecular events responsible for progressive diastolic dysfunction in the pressure overloaded heart.

B. REGULATION OF SERCA2 GENE EXPRESSION IN PRIMARY CULTURES OF NEONATAL MYOCYTES.

The signal transduction pathways involved in the down regulation of SERCA2 mRNA during pressure overload hypertrophy and CHF are undefined. It has been demonstrated that mechanical load is a very important trigger for induction of pressure overload LVH. A growing body of evidence has shown that PKC activation is involved in the gene regulation in *in vitro* model of cardiac hypertrophy induced by mechanical and hormonal stimuli. More recently, activation of PKC isoenzymes in cardiac tissues has been reported in several type of cardiac hypertrophy *in vivo* (review see Chapter II section G-2). Therefore, the present study investigated the role of mechanical activity and PKC

activation in the regulation of SERCA2 gene expression in primary cultures of neonatal rat ventricular myocytes.

1. Cell Culture Model.

A major difficulty in defining the signaling mechanisms responsible for alterations in cardiac myocyte gene expression during hemodynamic overload has been the limited ability to manipulate and control the complex *in vivo* physiology of the hypertrophic process. Thus, a critical advance in the study of the mechanisms of myocyte hypertrophy has been the development of techniques for the long-term culture of neonatal and adult cardiac myocytes. In comparison to the whole animal study, the use of cultured cells not only reduced the variables but also reduced animal usage and the time frame for the investigation. Accumulated evidence has demonstrated that several major features of pressure overload hypertrophy *in vivo* can be simulated in primary cultures of neonatal rat ventricular myocytes after stimulation with defined agonists (Chien et al., 1991). Therefore, it is likely that the hypertrophic response in both agonist-stimulated cultured cells and hemodynamically overloaded myocardium *in vivo* share common signaling mechanisms. However, it should be pointed out that data obtained from cultured neonatal cardiac myocytes may not always correlated with mature cardiac myocyte function *in vivo* because of the developmental differences as well from the effects of culture conditions.

To study the effects of mechanical load on cardiac hypertrophy, three types of cell culture model systems have been widely used. They are 1) spontaneous contraction of primary cultured neonatal rat ventricular myocytes as compared with contractile arrested cells (produced by either membrane depolarization or L-type Ca channel blockade) (McDermott et al., 1985; Marino et al., 1987; McDermott and Morgan, 1989; Samarel and Engelmann, 1991); 2) electrically stimulated contractile activity (Johnson et al., 1994); and 3) passive stretch of neonatal rat myocytes cultured on elastic membranes (Komuro et al., 1991). McDermott et al. (1985) have hypothesized that cellular shortening and lengthening during contraction and relaxation of neonatal rat myocytes attached to a rigid substratum may partially mimic the physical stimuli produced during both passive and active tension development in the intact tissue. In the present study, spontaneous contractile activity in primary cultured neonatal rat ventricular myocytes were arrested by Ca channel blockade, membrane depolarization or dissociation of E-C coupling. The various methods of producing contractile arrest not only inhibited the growth of neonatal cardiac myocytes in culture (Table 6) but also produced a genetic phenotype that was opposite to that found in cardiac hypertrophy *in vivo* (e.g. down-regulation of MHC- β and up-regulation of SERCA2 gene expression (Figure 11)).

Direct activation of PKC by PMA has been used by many investigators to study the signal transduction pathways involved in cardiac myocyte hypertrophy. In concurrence with others (Fuller and Sugden, 1989; Dunmonn et al., 1990; Samarel and Engelmann, 1991; Allo et al., 1991), the present study demonstrated that primary cultured neonatal rat

ventricular myocytes treated with PMA reproduced some features of the hypertrophic response to pressure overload *in vivo* such as increased protein content per cell without proliferation (as evidenced by increase in total protein and total protein/DNA ratio without an increase in DNA content) (Figure 15); and up-regulation of “fetal-type” contractile protein gene expression (e.g., MHC- β ; Figure 16). Therefore, PMA induced cardiac myocyte hypertrophy was used in the present study to examine the role of PKC activation in the regulation of SERCA2 gene expression.

2. Contractile Arrest by Verapamil Up-regulates SERCA2 Gene Expression

In primary cultured neonatal rat ventricular myocytes, 10 μ M of verapamil completely blocks Ca^{2+} entry via the L-type Ca^{2+} channel and eliminates phasic $[\text{Ca}^{2+}]_i$ transients and mechanical activity (Figure 29, Ojamaa et al., 1995). Inhibition of spontaneous contractile activity with verapamil caused significant myocyte atrophy and disproportionately reduced the intracellular content of a specific contractile protein, namely MHC- β (Samarel and Engelmann 1991, and this work). In the present study, contractile arrest by verapamil significantly up-regulated SERCA2 gene expression at both the mRNA and protein levels. The present study also indirectly addressed the relationship between SERCA2 gene expression and myocyte growth. Of note, the coordinate increase in SERCA2 mRNA and protein levels in verapamil-arrested myocytes was considerably greater than the decrease in total RNA and total protein per cell,

indicating that contractile arrest caused a specific increase in SERCA2 gene expression. Simultaneously, in the same experimental model, we (Bassani et al., 1994b) evaluated SR Ca uptake in both control and verapamil-treated cells by measuring the $t_{1/2}$ for $[Ca^{2+}]_i$ decline during twitches induced by cell depolarization in high K^+ and Ca^{2+} solution (K-twitch). Since the main mechanism responsible for cell relaxation and $[Ca]_i$ decline during a K-twitch is the SR Ca uptake. In this case the Na-Ca exchanger is inhibited and the other mechanisms (mitochondrial uniporter and sarcolemmal Ca pump) are much too slow to compete with the SR Ca ATPase in removing Ca from the cytosol (Bassani et al, 1994a). As summarized in Figure 30, verapamil arrest induced a marked increase in SR Ca^{2+} uptake rate in parallel with the increases in SERCA2 mRNA and protein levels. The simplest interpretation of this result is that there is an increase in the number of SR Ca pumps, with concomitant increase in Ca transport by the SR Ca pump. These results indicate that SR Ca ATPase protein levels are regulated at least partly at pretranslational levels.

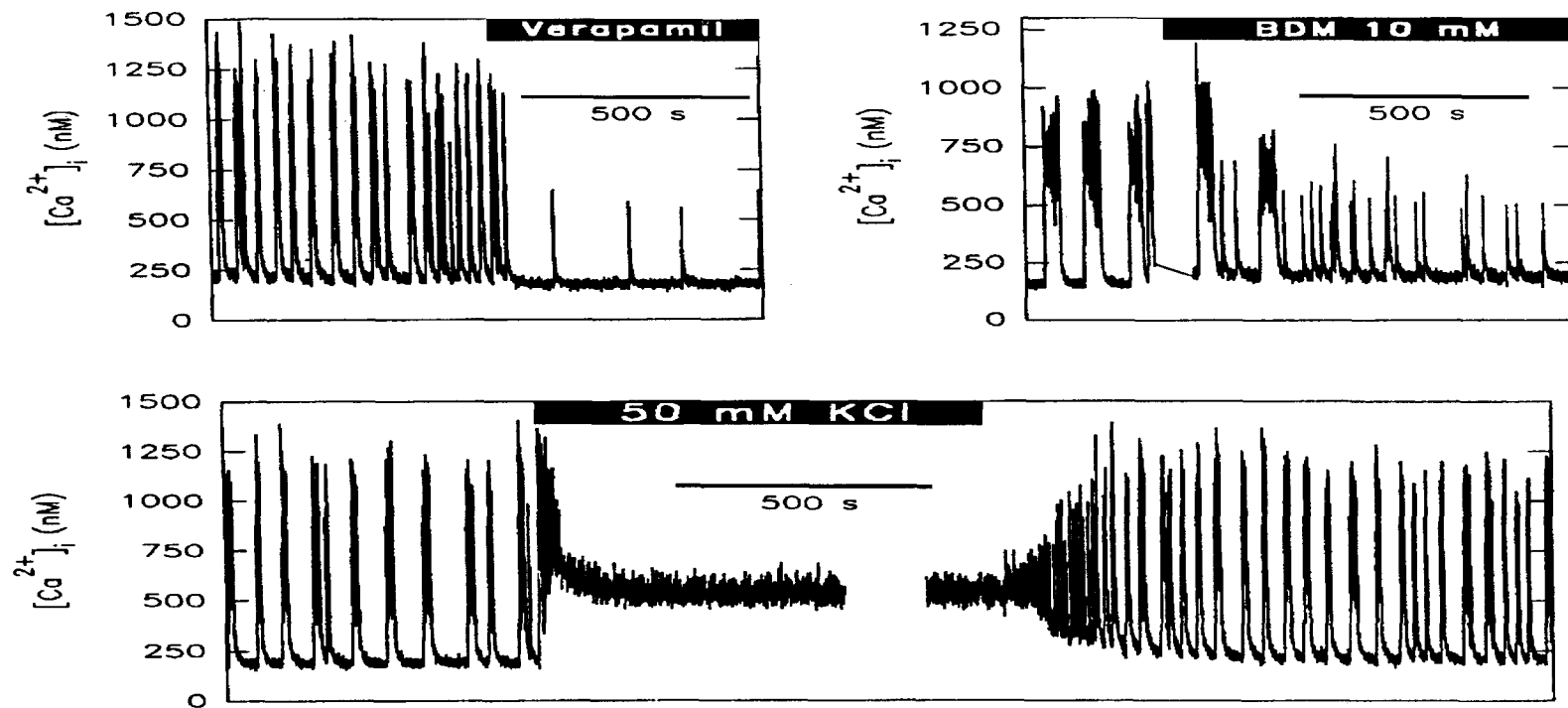


Figure 29: Measurement of $[Ca^{2+}]_i$ in verapamil, BDM and KCl treated cells. Neonatal rat ventricular myocytes were maintained in standard growth medium for 48 h. The cells were loaded with the fluorescent indicator, fura-2 (2 μ M for 2.5 hr at room temperature). A Perkin-Elmer LS 50B fluorescence spectrophotometer was used to measure fura-2 fluorescence from populations of myocytes. Calibration of fura-2 fluorescence in terms of $[Ca^{2+}]_i$ routinely utilized solutions of known Ca^{2+} concentration to construct a standard curve; a lookup table was then prepared for analysis of fluorescence ratio. Verapamil = 10 μ M verapamil. — represent 500 seconds time interval. (Data are from Ojamaa et al., 1995).

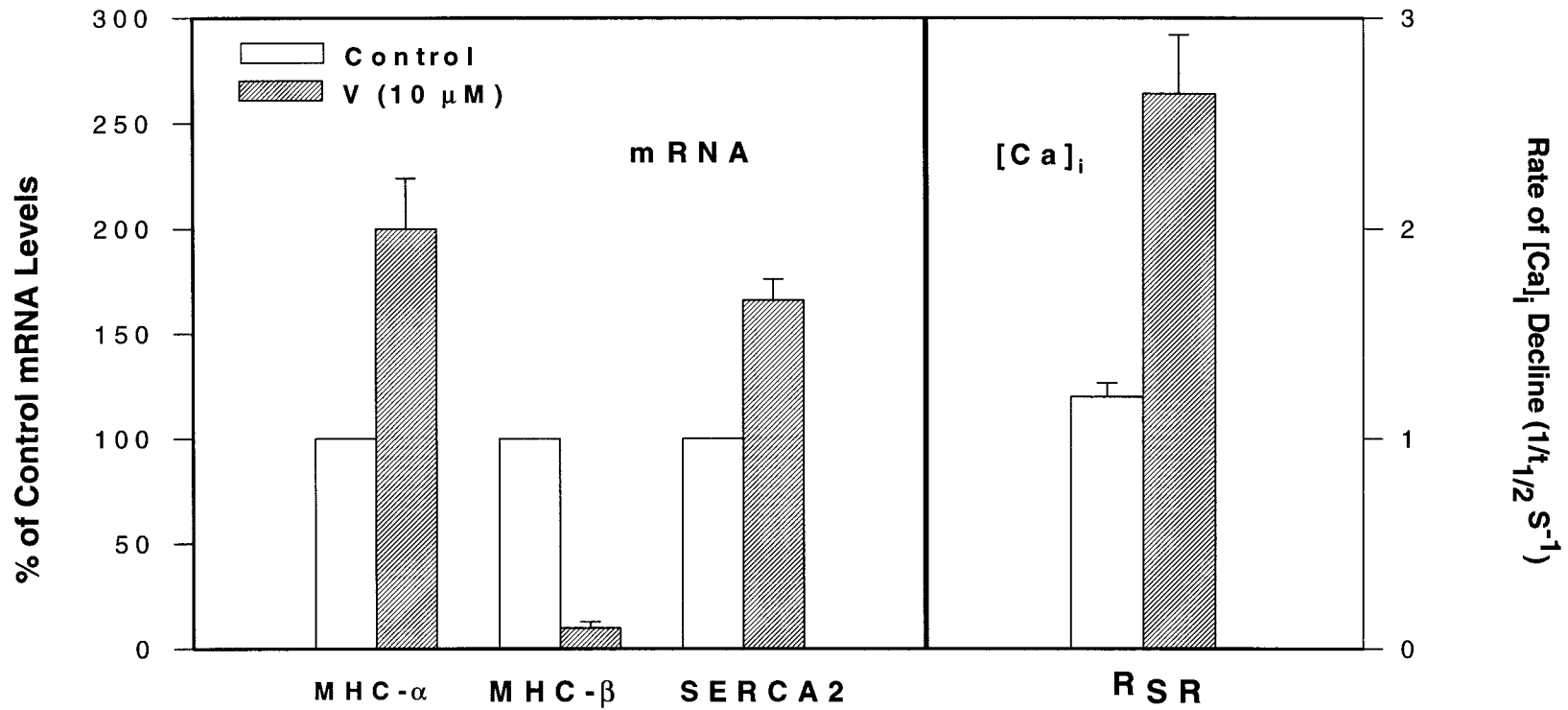


Figure 30: Effect of verapamil arrest (V) on SERCA2 mRNA and SR Ca uptake in cultured neonatal rat ventricular myocytes. Neonatal rat ventricular myocytes were maintained (48 h) in standard culture medium (Control) or medium supplemented with 10 μ M verapamil (V). The cells were harvested for Northern blotting analysis. The message levels are normalized to GAPDH mRNA and plotted as percent of the value in control cells. The cells grown in cell chambers were used for Ca transient measurements. The index of $[Ca]_i$ decline via SR Ca^{2+} pump (R_{SR}) is the inverse of the steady state value of $t_{1/2}$ for the K-twitch. (Data are from Bassani et al., 1994).

3. Changes in Contractile Activity Rather Than Alterations in $[Ca^{2+}]_i$ Regulate SERCA2 Gene Expression.

Verapamil, KCl and BDM were used in the present study to determine whether changes in mechanical activity or alterations in $[Ca^{2+}]_i$ regulate SERCA2 gene expression. All three of these agents inhibit spontaneous contractile activity of neonatal myocytes. However, their effects on $[Ca^{2+}]_i$ are very different. In neonatal rat ventricular myocytes, 10 μ M verapamil completely blocks Ca^{2+} entry and eliminates phasic $[Ca^{2+}]_i$ transients. In contrast, a high concentration (50 mM) of KCl induces membrane depolarization and causes a tonic rise in $[Ca^{2+}]_i$ (Figure 29). BDM modestly reduced the amplitude of the $[Ca^{2+}]_i$ transient (Figure 29). The present study showed that verapamil, KCl (50 mM) and BDM (10 mM) completely arrested the cells, down-regulated MHC- β and up-regulated SERCA2 mRNA levels despite the significant differences in $[Ca^{2+}]_i$. These data indicate that contractile activity rather than changes in $[Ca^{2+}]_i$ levels regulate SERCA2 gene expression.

4. PKC Activation Down-Regulates SR Ca ATPase and Decreases SR Ca Uptake

a). PMA-induced myocyte hypertrophy. Recent studies have demonstrated that pressure overload hypertrophy is associated with changes in the activity of PKC or PKC isoenzymes (Makita and Yasuda, 1990, Gu and Bishop, 1994). However, it is difficult to define the signaling mechanisms responsible for alterations in cardiac myocyte gene

expression during hemodynamic overload in an *in vivo* study. In the present study, I demonstrated that primary cultures of neonatal rat ventricular myocytes treated with PMA reproduced some features of the hypertrophic response to pressure overload *in vivo*. PMA treatment: (a) produced myocyte hypertrophy as evidenced by an increase in total protein and total protein/DNA ratio (Figure 15); (b) up-regulated “fetal-type” contractile protein gene expression (e.g., MHC- β ; Figure 16); and (c) down-regulated SERCA2 mRNA and protein levels leading to prolonged $[Ca^{2+}]_i$ decline (Qi et al. unpublished data). Previous studies have documented that prolonged exposure to PMA (48-96 h) increased total protein (Allo et al, 1991, Dunnmon et al, 1990) and total RNA (Allo et al., 1991) content, and increased cell size in culture. Therefore, PMA induced myocyte hypertrophy has been used in the present study to investigate the role of PKC in the regulation of SERCA2 gene expression during pressure overload hypertrophy.

b). Role of PKC isoenzymes in SERCA2 down-regulation. Although PMA treatment produced myocyte growth and down-regulation of SERCA2 mRNA and protein levels, an obvious question raised by the present results is whether these events were indeed due to the activation of one or more PKC isoenzymes. Cultured neonatal rat ventricular myocytes express the Ca^{2+} -dependent isoenzymes PKC- α and PKC- β , the Ca^{2+} -independent isoenzymes PKC- ϵ and PKC- δ , as well as the atypical PKC- ζ (Mochly-Rosen et al, 1990, Puceat et al, 1994, Rybin and Steinberg, 1994). It has been demonstrated that PMA induces the rapid translocation of cytosolic PKC- α , β , ϵ , and δ to the particulate fraction of neonatal myocytes, with little or no effect on PKC- ζ (Johnson

et al., 1995, Rybin and Steinberg, 1994). Thus, PMA stimulation produces a rather nonselective activation of multiple myocyte PKC isoenzymes. However, isoenzyme-dependent differences in substrate specificity might produce differences in functional responsiveness. A recent study (Puceat et al., 1994) has shown that PKC- ϵ may be responsible for agonist-stimulated phosphorylation of the 85 kDa MARCKS protein, an endogenous PKC-specific substrate. In contrast, agonist-induced *c-fos* expression is likely to be mediated by PKC- α or δ (Puceat et al., 1994). Additional studies will be required to determine which PKC isoenzyme(s) is responsible for the observed changes in SERCA2 gene expression. However, my results indicating that relatively specific inhibitors of PKC block the PMA-induced down-regulation of SERCA2 mRNA levels (Figure 20) is strong evidence that these changes were in fact related to the activation of one or more PKC isoenzymes, and not the result of a nonspecific effect of the drug.

c). Activation vs down-regulation of PKC by PMA. It should also be pointed out that although PMA activates the PKC isoenzymes expressed in neonatal rat cardiac myocytes, the drug also causes the time-dependent loss of some PKC isoenzymes from the cell. Johnson and Mochly-Rosen (1995) and Puceat et al (1994) have shown that PMA causes a transient activation and subsequent down-regulation of PKC- α , β , ϵ , and δ in these neonatal myocyte cultures but each isoenzyme is down-regulated at a distinctively different rate (Puceat et al, 1994, Johnson and Mochly-Rosen, 1995). As many of the experiments in this study involved the continuous exposure of myocyte cultures to PMA for up to 72 h, it is possible that some of the observed effects were actually due to down-

regulation (rather than activation) of PKC isoenzyme activity by PMA. However, the time course of PMA incubation with neonatal rat myocytes shows that there were no significant increases in SERCA2 mRNA levels during incubation with PMA. Furthermore, this study also showed only a brief exposure (30 min) to PMA was sufficient to produce a significant decrease in SERCA2 mRNA levels over a subsequent 48 h period (Figure 19). Johnson and Mochly-Rosen (1995) have demonstrated that treatment of neonatal myocytes with 100 nM PMA for 30 min caused the marked translocation of all of the PMA-responsive, PKC isoenzymes, but caused only a partial reduction in total PKC- α , β , δ . Total PKC- ϵ content (the major PKC isoenzyme present in cultured neonatal rat myocytes) was not reduced during this time period. In fact, activated, membrane-bound PKC- ϵ persisted for up to 8 h during continuous exposure of neonatal myocytes to 100 nM PMA (Puceat et al., 1994). Thus, these results indicating that only a brief exposure to PMA was sufficient to decrease SERCA2 mRNA levels strongly suggest that down regulation of SERCA2 mRNA was the result of activation, rather than down-regulation of one or more PKC isoenzymes.

To further ascertain the role of PKC in the regulation of SERCA2 gene expression, two PKC inhibitors (staurosporine and chelerythrine) were used in this study. Staurosporine, which is the most potent general inhibitor of protein kinases, has been widely used as a PKC inhibitor (Tamaoki et al, 1986, 1991). Although, staurosporine at high concentrations inhibits other protein kinases including protein kinase A, low concentration of staurosporine have been used as a relatively specific PKC inhibitor

(Kageyama et al, 1992, Allo et al, 1992). A very recent study (Budworth and Gescher, 1995) showed that staurosporine has high potency against membrane-derived PKC. Therefore, in the present study, a low concentration (10 nM) of staurosporine was used and it blocked the PMA induced down regulation of SERCA2 mRNA levels. A more specific PKC inhibitor chelerythrine (Herbert et al., 1990) was also used in the present study and it showed a similar result as staurosporine. These results along with the similarity of results obtained by acute or chronic PMA exposure, strongly indicate that PMA induced down regulation of SERCA2 mRNA levels was the result of activation, rather than down-regulation of one or more PKC isoenzymes.

d). Changes in SR Ca ATPase content and function in PMA treated cells. In the present study, I demonstrated that reduced SERCA2 mRNA levels were accompanied by a decrease in SR Ca ATPase protein. This indicates again that SR Ca ATPase protein levels are at least partly regulated at a pre-translational level. Similar coordinate decreases in SERCA2 mRNA and protein levels were observed by others in the hypertrophied and failing adult rat heart (Komuro et al., 1989 and Studer et al., 1994). It should be pointed out, however, that the Western blotting technique used to analyze the number of SR Ca pumps reflected the concentration of this protein relative to the total protein pool of the cultured cells. However, the reduction in SERCA2 protein (53%) was considerably greater than the increase in total protein or total protein/DNA ratio (26 and 15 %, respectively). These results indicate that the reduced number of SR Ca²⁺ pumps resulted from a specific decrease in the expression of SERCA2 rather than dilution. To

further prove this conclusion, $[Ca^{2+}]_i$ decline during K-twitch was measured. As seen in Figure 31, the time to peak $[Ca^{2+}]_i$ and the rate of decline of $[Ca^{2+}]_i$ was slower in PMA-treated cells (Qi et al., unpublished data). These data are in excellent agreement with this conclusion.

In summary, the present study demonstrates that activation of PKC by PMA significantly decreased SERCA2 mRNA and protein levels in primary cultures of neonatal rat ventricular myocytes. SR Ca ATPase levels are regulated at least partly at a pretranslational level. The reduction of SERCA2 mRNA and protein concentration accounted for the decrease in SR Ca uptake and ultimately contributed to the impaired decline in the $[Ca^{2+}]_i$ transient of the neonatal cardiac myocyte. Thus, these data suggest that PKC activation may play an important role in the regulation of SERCA2 gene in the hypertrophied and failing heart.

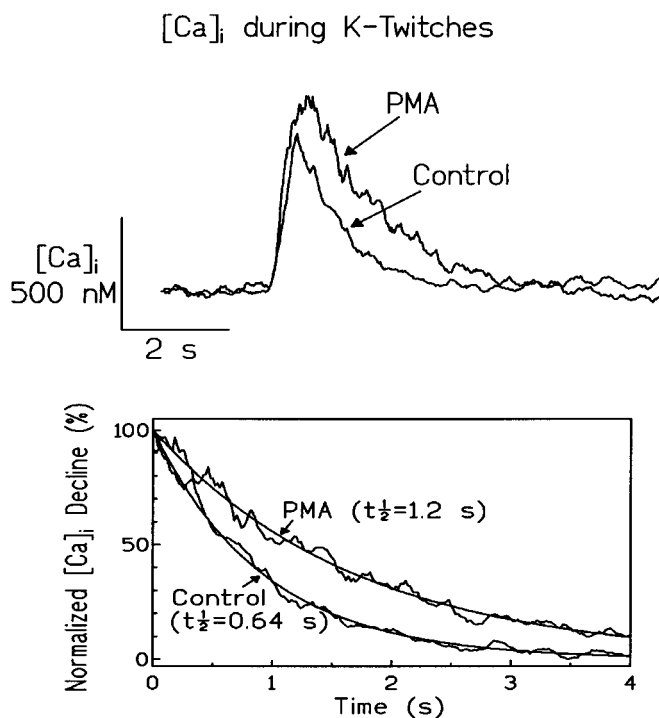


Figure 31: SR Ca pump in control and PMA-treated neonatal rat ventricular myocytes. The contribution of SR Ca ATPase activity to the decline of the $[Ca^{2+}]_i$ transient in indo-1 loaded myocytes was evaluated by measuring the steady-state values of $t_{1/2}$ for $[Ca^{2+}]_i$ decline during twitches induced by cell depolarization (K-Twitch), which were obtained by brief (100 ms) application of high K solution during perfusion with 0 Na, 0 Ca (where Na-CaX is prevented). Measurements were obtained after a series of K-twitches at 0.5 Hz to load the SR to a steady state level. The increased time to peak and slowed $[Ca^{2+}]_i$ decline in the PMA-treated cell in the upper panel are typical and consistent with slowed SR Ca uptake. In the lower panel, the data were normalized to peak $[Ca^{2+}]_i = 100\%$, re-plotted, and curve fitted with exponential function to demonstrate the difference in $t_{1/2}$ for $[Ca^{2+}]_i$ decline between control and PMA-treated cells. (Data are from Qi et al., 1994 b).

C. TRANSCRIPTIONAL VS. POST-TRANSCRIPTIONAL REGULATION OF SERCA2 GENE EXPRESSION.

1. Regulation of SERCA2 Gene Expression at the Transcriptional Level.

As discussed previously, verapamil and PMA regulate SERCA2 gene expression at a pretranslational level. In the present study, a relatively small fragment of the rat SERCA2 promoter (556 bp) was used to regulate the expression of a luciferase reporter in order to analyze the effects of verapamil and PMA on SERCA2 gene transcription. The results show that verapamil treatment did not change SERCA2 promoter activity (Figure 27). In contrast, despite a significant reduction in SERCA2 mRNA and protein levels, PMA treatment appeared to increase SERCA2 promoter activity as measured in a transient expression assay. A similar promoter-reporter gene transient transfection assay has been used to analyze muscle-specific transcription (Fisher et al., 1993, Rohrer et al., 1991, Sukovich et al., 1993, Zarain-Herzberg et al., 1990), and thyroid hormone responsiveness of the rat (Hartong et al., 1994, Rohrer et al., 1991) and rabbit (Zarain-Herzberg et al., 1994) SERCA2 genes. This upstream promoter region contains sufficient regulatory sequences to confer transcriptional activation in muscle cells (Fisher et al., 1993, Rohrer et al., 1991, Sukovich et al., 1993) and transactivation by T₃ (Hartong et al., 1994, Rohrer et al., 1991, Zarain-Herzberg et al., 1994). However, it is conceivable that regulatory elements further upstream from the transcriptional start site may have altered PMA responsiveness of the reporter gene to indicate down-regulation rather than modest

activation, as has been suggested by Dillmann et al (1992). The same argument can be made regarding the verapamil responsiveness of the reporter gene since a “contractile element” may also be located further upstream. Nevertheless, it appears unlikely that the rapid reduction in SERCA2 mRNA levels in PMA-treated cells can be explained by alterations in endogenous gene transcription rate.

2. Regulation of SERCA2 Gene Expression by Alterations in Message Stability.

In addition to the level of transcription, I hypothesized that SERCA2 gene expression was regulated by changes in the SERCA2 mRNA stability. To determine if verapamil or PMA can alter mRNA stability, a pharmacological inhibitor of mRNA synthesis, Act-D, was used in the present study to define the half-life of the SERCA2 mRNA under control and experimental conditions. The concentration of Act-D (5 μ g/ml) used in this study has been previously shown to inhibit all transcriptional activity in primary cultured neonatal rat ventricular myocytes (Kamitani et al, 1992, LaPointe and Sitkins, 1993). Act-D was added after 24 h incubation with various agents, then the cells were harvested for the measurement of SERCA2 mRNA levels at different time points. In this study, 18S rRNA levels were used for normalization of SERCA2 mRNA levels since this study and others have shown that GAPDH mRNA levels were decreased (Tsujino et al, 1994) and 18S rRNA levels were relatively stable (Harrold et al., 1991) during brief Act-D treatment.

Based upon the results depicted in Figure 28, verapamil stabilized the SERCA2 mRNA transcript. This may be a contributing factor to the up-regulation of SERCA2

mRNA levels by verapamil. In contrast, a major effect of PMA treatment on SERCA2 gene expression is to destabilize the relatively long-lived SERCA2 mRNA transcript. The nearly 3-fold reduction in mRNA half-life must contribute to the rapid decline in SERCA2 mRNA levels, counteracting the modest increase in SERCA2 gene transcription (Figure 32). Interestingly, Choi et al (1991) have shown that phorbol esters increase the turnover of α -cardiac and α -skeletal actin mRNA's in chick skeletal muscle myotubes, perhaps by affecting the synthesis of a protein responsible for increased degradation of specific mRNA's. Additional studies will be required to determine if PKC-dependent signaling pathways are responsible for regulating SERCA2 mRNA stability, and to analyze its relevant to pressure overload induced down regulation of SERCA2 during hemodynamic overload *in vivo*.

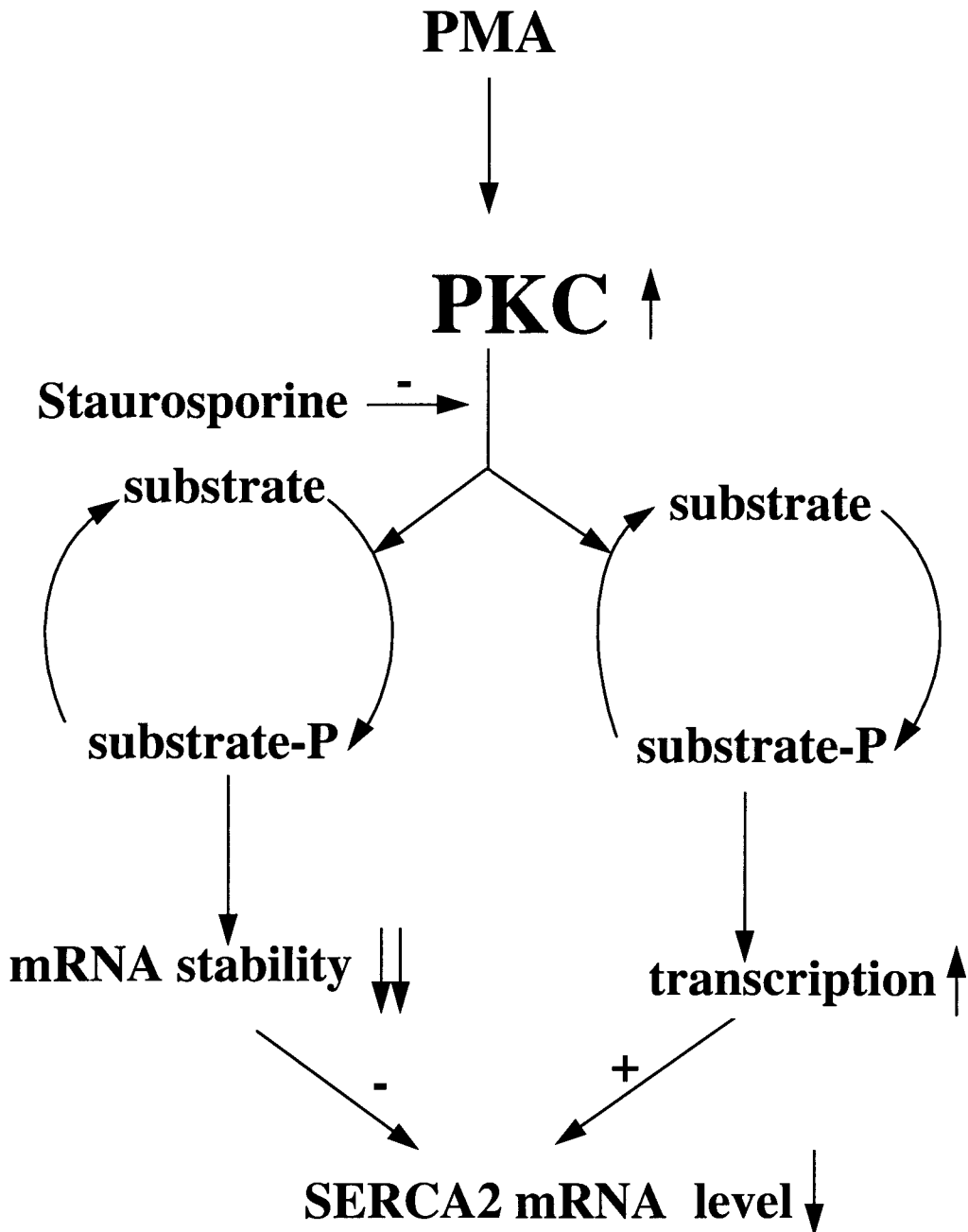


Figure 32. Hypothesized mechanisms for PMA induced down-regulation of SERCA2 mRNA levels.

D. SUMMARY:

In this study, I produced chronic pressure overload LVH by coarctation of the abdominal aorta and addressed the hypothesis that slowed myocardial relaxation observed in the chronically pressure-overloaded LV resulted from reduced numbers of SR Ca^{2+} pumps. Although I clearly demonstrated that pressure-overload induced LVH was associated with abnormalities in myocardial relaxation (as indicated by changes in normalized maximum $-\text{dP}/\text{dt}$ and τ), these hemodynamic changes were not initially associated with a reduction in the number of SR Ca^{2+} pumps. Reduced SERCA2 protein levels were not observed in this model of pressure overload until myocardial function (as measured by the LVEDP) was significantly impaired. Increased LV tissue collagen content was correlated with the cardiac relaxation abnormality that was observed in 8 wk banded rats, indicating that ECM remodeling may be responsible for the initial relaxation abnormality in compensated, pressure overload LVH. I speculated that other factors such as a decrease in SR Ca^{2+} ATPase activity (without a reduction in the number of SR Ca^{2+} pumps) may also contribute to the relaxation abnormality in the early stage of the pressure overload LVH. Nevertheless, down-regulation of SERCA2 mRNA levels preceded the deterioration of cardiac function suggesting that down-regulation of the SERCA2 gene may be a useful marker to predict the onset of overt cardiac dysfunction during the development of pressure overload LVH. Furthermore, down-regulation of SERCA2 mRNA and protein levels in 16 wk banded rats may be an important

contributing factor in the progressive deterioration in LV function during the transition from compensated pressure overload LVH to heart failure.

The molecular mechanisms which may be involved in the regulation of SERCA2 gene expression during pressure overload LVH and heart failure were investigated in a cultured neonatal rat ventricular myocyte model. Firstly, the role of mechanical activity in the regulation of SERCA2 gene expression was studied by arresting cells with verapamil, KCl and BDM. Verapamil arrest increased SERCA2 mRNA and protein levels, indicating that verapamil induced changes in SR Ca ATPase content are regulated at pretranslational levels. SERCA2 mRNA levels were also up-regulated in KCl and BDM arrested cells, suggesting that changes in contractile activity rather than changes in $[Ca^{2+}]_i$ regulate SERCA2 mRNA levels. Secondly, the role of PKC in the regulation of SERCA2 gene expression was also examined. The PKC activator PMA induced cardiac myocyte hypertrophy and caused down-regulation of SERCA2 mRNA and protein levels. The effect of PMA-induced down-regulation of SR Ca ATPase were blocked by PKC inhibitors. These results indicate PKC activation down-regulates SERCA2 mRNA levels. These data suggest that PKC activation may play an important role in the down-regulation of the SERCA2 gene during pressure overload. Finally, the transient transfection assay and Act-D message stability assay demonstrate that the changes in SERCA2 mRNA levels by verapamil and PMA were not primarily controlled at the transcriptional level. Changes in SERCA2 message stability are responsible for the

changes in SERCA2 mRNA level in these cell culture models of myocyte atrophy and hypertrophy.

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C. Review

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the committee with reference to content and form.

This dissertation is therefore accepted in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

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